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14. ABSTRACT This research project focuses on the role of macrophages in early dissemination and dormancy. We hypothesized that macrophages are actively recruited by pre-malignant ErbB2 overexpressing cancer cells and that these intra-epithelial macrophages then produce factors that induce an EMT and thereby facilitate early dissemination. We further hypothesized that bone marrow (but not lung) macrophages produce TGFβ2, BMP7 and other factors that instruct DCCs to enter dormancy. In funding year 1 we provided evidence that early ErbB2+ lesions, but not healthy mammary tissue, produced CCL2 in an NFκB dependent manner and thereby recruited resident macrophages inside the duct. Intra-ductal macrophages secreted Wnt1 and thereby induced an EMT in early ErbB2+ cancer cells. Depletion of macrophages - but only before overt advanced tumors appeared - drastically reduced early dissemination and surprisingly the onset of metastasis even after macrophages repopulated the overt tumor tissue. We also proposed that cancer cells that disseminated early play a long-term causal role in metastasis development. In funding year 2 we demonstrated that bone resident macrophages inhibit proliferation of disseminated tumor cells and are responsible for less efficient metastasis formation. When bone resident macrophages are co-injected with breast cancer cells in an experimental metastasis assay in vivo, this significantly reduced lung metastasis formation, demonstrating that resident macrophages can decide over the fate of disseminated tumor cells. We further revealed that bone resident macrophages produce TGFβ2, a factor known to be able to induce dormancy. In contrast, lung resident macrophages produce only low amounts of TGFβ2 and seem to inhibit TGFβ2 production in the lung microenvironment. Overall, these results demonstrate that tissue resident macrophages are highly specific in their function and can decide over the fate of disseminated tumor cells. Overall, the results of this grant have shed light on the mechanism of early dissemination and have revealed a mechanism how macrophages might instruct disseminated tumor cell dormancy in the bone marrow.		

15. SUBJECT TERMS early dissemination, EMT, DCIS, macrophages, tumor microenvironment, metastasis, dormancy, bone metastasis, lung metastasis					
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1. INTRODUCTION

Most breast cancer patients die from metastatic disease that are mostly incurable. Metastases can occur years or decades after removal of the primary tumor, suggesting there is a window of opportunity to prevent their outgrowth. Additionally, the field of metastasis research has been challenged by the finding that dissemination does not only occur from late stage invasive tumors but can already occur during early pre-invasive breast cancer stages as revealed by large cohort patient studies [1-4] and studies with spontaneous mouse tumor models [5]. This might lead to an increased heterogeneity of disseminated cancer cells (DCCs) that colonized target organs during different time points of progression and contribute to metastasis. Macrophages were implicated in regulating dissemination (local invasion migration and intravasation) in overt tumors with pathologically defined invasive characteristics [6, 7] and are important for growth of macro-metastases [7, 8], however whether they are involved in early dissemination and the regulation of growth of disseminated tumor cells has never been studied.

In this research project we **hypothesized** that macrophages are actively recruited by pre-malignant ErbB2 overexpressing cancer cells and that these intra-epithelial macrophages then produce factors that induce an EMT and thereby facilitate early dissemination. We further hypothesized that bone marrow (but not lung) macrophages produce TGF β 2, BMP7 and other factors that instruct DCCs to enter dormancy.

In funding year 1 we provided evidence that early ErbB2+ lesions, but not healthy mammary tissue, produced CCL2 in an NF κ B dependent manner and recruited intra-ductal macrophages, that secrete Wnt1 and thereby induce an EMT in the early ErbB2+ cancer cells. Depletion of macrophages before overt advanced tumors appeared drastically reduced early dissemination and surprisingly, the onset of metastasis even after macrophages repopulated the overt tumor tissue. Importantly, humans with DCIS lesions, a very early stage of breast cancer, that contained macrophage+/E-Cadherin^{lo} microenvironments frequently had disseminated cancer cell (DCCs) in the bone marrow. We reveal that resident macrophages can promote early dissemination explaining how early cancer spread might proceed in breast cancer patients. We also propose that eDCCs play a long-term causal role in metastasis development. These results were mostly collected in funding year 1 and are presented in the **manuscript in the appendix**. **In funding year 2 we provide evidence** that lung resident M Φ s induce a proliferation program whereas BM resident M Φ s induce a dormancy program in DCCs. We compared the expression levels of dormancy inducing factors previously described in our lab (TGF β 2, BMP7) and found that BM resident M Φ s produce high levels of TGF β 2 whereas lung resident M Φ s produce low levels and seem to further inhibit TGF β 2 production in the lung microenvironment. We further demonstrated that when bone resident macrophages are co-injected with breast cancer cells in an experimental metastasis assay in vivo, lung metastasis formation was significantly reduced. This demonstrates that resident macrophages can decide over the fate of disseminated tumor cells.

2. KEY WORDS

early dissemination, EMT, DCIS, macrophages, tumor microenvironment, metastasis

3. ABBREVIATIONS AND NOMENCLATURE

We previously referred to cells in very early stage breast cancer lesions as “pre-malignant epithelial cells” (PM-MECs). In the manuscript we changed this nomenclature to “early cancer cells” (eCCs) to address the possibility that there might be a subpopulation of invasive cancer cells within early lesions. Consequently, we refer to “disseminated cancer cells” (DCCs). In the manuscript we also use the synonym Her2 instead of ErbB2. However this is the same protein and mouse model.

EMT – epithelial to mesenchymal transition

DCIS – ductal carcinoma in situ

CC – cancer cell
eCC – early cancer cell
CCC – circulating cancer cell
DCC – disseminated cancer cell

4. ACCOMPLISHMENTS.

Specific Aim 1. To determine the role of MΦs in early dissemination.

Objectives. We will determine which cytokines produced by ErbB2^{hi} / p38^{low} mammary ducts recruit MΦs. We will investigate how intra-epithelial MΦ induce an EMT in ECCs. We will characterize a profile of an early dissemination microenvironment that we will confirm using 3D *in vitro* experiment. We will further confirm the significance of our findings using human tissue microarrays to identify those genes whose expression indicate an early dissemination microenvironment.

Results. All experiments suggested in SA1 have been successfully performed in funding year 1. A manuscript was submitted in October 2015 of which we had provided a copy in the progress report of funding year 1. The manuscript is still under revision and to address reviewer's comments, we performed several additional experiments. We now attach the revised manuscript. The additional experiments that were included in the revised manuscript version are as follows:

- To provide a better overview of the early stages of breast cancer that our study focuses on, we included HE stainings of healthy wild type and pre-malignant mammary glands and invasive tumors (Fig.1A-C).
- We had shown that pre-malignant mammary epithelial cells next to intra-epithelial macrophages show disorganized and reduced E-Cadherin signal. E-Cadherin is an epithelial cell marker and its loss indicates an epithelial to mesenchymal transition. To strengthen our conclusion that intra-epithelial macrophages induce an EMT, we analyzed β-Catenin. We found that early cancer cells adjacent to intra-epithelial macrophages contain (Fig.2E-G), strengthening our conclusion that intra-epithelial macrophages induce an EMT. We had performed a mass cytometry (CyTOF) analysis of mammary gland macrophages. We now expanded this analysis, including more animals in the experiment (Fig.5). Additionally, the CyTOF analysis was complemented by in situ stainings of CD206 in wild type and pre-malignant mammary glands as well as invasive tumors (Fig.5K-N).
- We had identified that CCL2 produced by HER2+ early cancer cells was responsible to attract macrophages into pre-malignant ducts. We now confirmed those findings by performing stainings revealing the presence of CCL2 next to HER2+ early cancer cells and CCR2+ macrophages (Fig.6C). Additionally, we had blocked the receptor for CCL2, CCR2, systemically. To address whether macrophages recruited into the duct were circulation derived or resident tissue derived, we now also blocked CCR2 locally by injecting the inhibitor into the mammary fat pad on one side and treated the contra-lateral mammary gland with vehicle control. We found that those glands injected with the CCR2 inhibitor contained less ducts with intra-epithelial macrophages compared to the contra-lateral control treated glands (Fig.6N,O). If CCR2 inhibition was affecting circulating macrophage precursors, then local injection of the CCR2 inhibitor should not affect macrophage localization. The results therefor indicate that intra-epithelial macrophages are derived from resident tissue macrophages.

Specific Aim 2: To determine how target organ-specific MΦ dictate DCC fate.

Objectives. To determine how lung resident MΦs induce a proliferation program and how BM resident MΦs induce a dormancy program in DCCs. We will compare expression profiles of lung and BM resident MΦs and compare expression levels of dormancy inducing factors previously described in our lab (i.e. TGFβ2, BMP7). We will validate identified factors responsible for the induction of a dormancy program *in vitro* and *in vivo*.

Task 1: Direct effect of lung and BM and lung MΦs on tumor cell proliferation

We completed *SA2.1a* and confirmed the adverse effect of lung and BM MΦs on tumor cell proliferation *in vitro* (Fig.2.1). We included a 2D proliferation assay and confirmed that CM from lungs stimulates 4T1 cell proliferation in 2D but this is reversed when macrophages are depleted. In contrast, CM from BM inhibits proliferation but only when BM contains macrophages. This method will provide a good and simple assay to screen for the effect of macrophage derived factors on dormancy (see SA2.3). Using a mammosphere assay, we could confirm the specific effect of lung and BM CM from macrophage depleted or control treated animals (Fig.2.1B-F). All these experiments have been repeated 3 times with high reproducibility and are thus sufficient for publication.

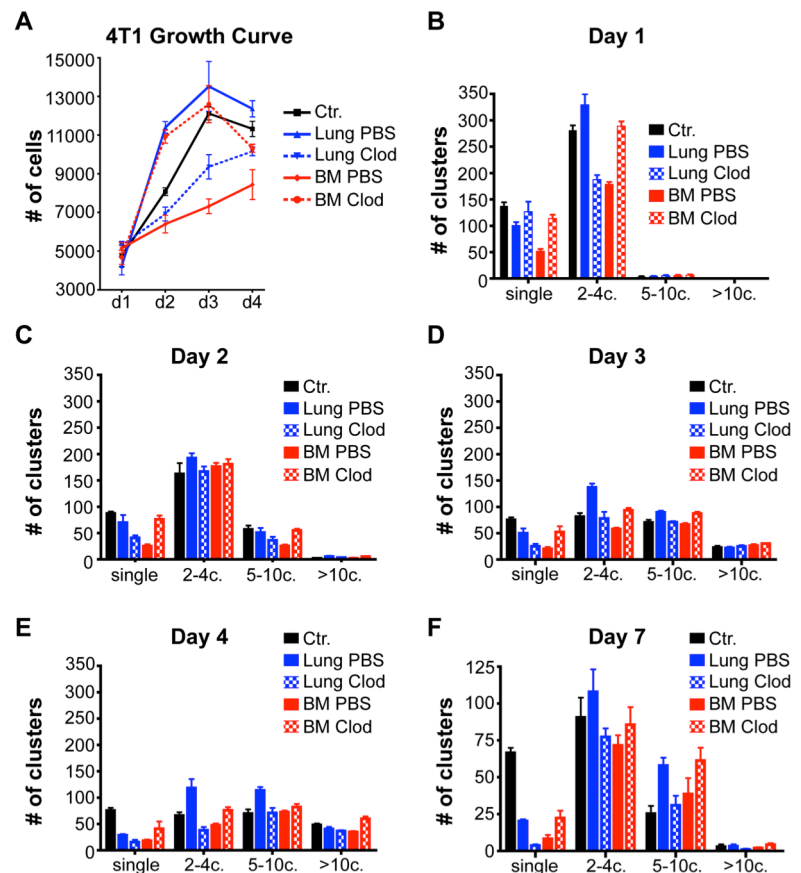


Fig.2.1: Effect of CM from Lung and BM on growth of 4T1 cells in 2D (A) and 3D (B-F). To deplete macrophages from wild type mice, these were treated with Clodronate liposomes (Clod). PBS liposomes (PBS) were used as control treatment. CM from macrophage containing (PBS) or macrophage depleted (Clod) Lung and BM cells was made and added daily to 2D and 3D assays of 4T1 cells. **A:** CM from lungs stimulates 4T1 cell proliferation in 2D but this is reversed when macrophages are depleted. Contrary, CM from BM inhibits proliferation but only when BM contains macrophages. **B-F:** CM was added to 4T1 grown as mammospheres and numbers of single cells and cell clusters was quantified on day 1-4 and day 7. BM CM reduced both single cells as well as proliferating cell clusters. 3 technical replicates, N=3 independent experiments.

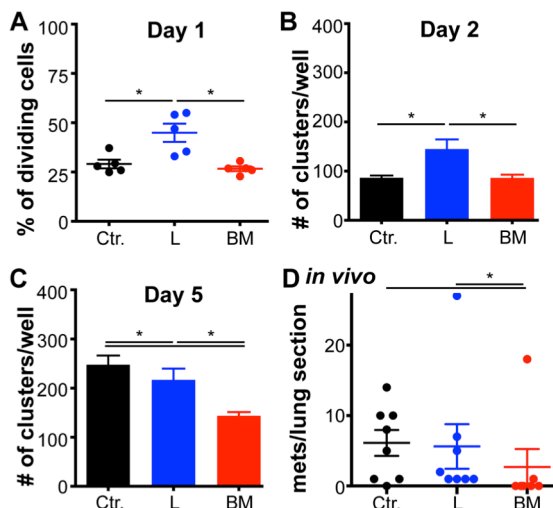


Fig.2.2: Direct effect of lung and BM macrophages on growth of 4T1 cells in 3D in vitro and in vivo. 4T1-GFP cells were seeded in matrigel either alone or mixed with primary lung or bone marrow macrophages. Dividing 4T1 cells were quantified after 1 day and clusters >2cells were quantified on day 2 and 5. While lung macrophages initially stimulated proliferation (A,B), BM macrophages had a long term growth inhibitory effect (C). 4T1-mCherry cells were injected into the tail vein of wild type mice either alone or in a 1:1 ratio with either lung or BM resident macrophages. 4T1 metastases were quantified after 7d. There were significantly less metastases detected when 4T1 were co-injected with BM macrophages compared to lung macrophages or no coinjection (D). Every dot represents one mouse, two independent experiments combined.

As further proposed, we tested the direct effect of lung and BM resident MΦs on cancer cell proliferation by performing a direct 3D co-culture (Fig.2.2A-C). We found that while lung macrophages initially stimulated proliferation (Fig.2.2A,B), BM macrophages had a long term growth inhibitory effect, resulting in less 4T1 cancer cell cluster formation (Fig.2.2C).

We further completed *SA2.1b* and We then confirmed these findings in an experimental metastasis vivo and co-injected 4T1-mCherry cancer cells with either lung or BM

resident MΦs. The addition of BM MΦs significantly reduced metastasis formation compared to co-injection with lung MΦs or injection of 4T1 cancer cells alone (Fig.2.2D).

Task 2: Expression of dormancy-inducing factors in lung and BM MΦs.

As proposed in SA2.2a, we determined TGFβ2 produced by resident macrophages from either lung or BM (**Fig.2.3**). We found that TGFβ2 levels were higher in total BM and primary BM MΦs CM compared to total lung and lung MΦ CM. Additionally, when MΦs had been removed from the organ cell suspensions by FACS sorting (Fig.2.3 “all cells but macs”), this significantly increased TGFβ2 levels in lung suspension and significantly reduced TGFβ2 levels in BM suspension. Similarly, TGFβ2 levels increased in lung suspension but decreased in BM suspensions when macrophages were depleted in vivo using clodronate liposomes. This provided a quantitative confirmation of our preliminary data analyzing TGFβ2 levels by Western Blot (see initial grant application. As further proposed in SA2.2a, we also analyzed BMP7 levels by ELISA but did not find significant differences in BMP7 secretion by BM or lung MΦs (data not shown).

Previous work from our lab has shown that high TGFβ2 induce dormancy in disseminated cancer cells [9]. We could confirm that 4T1 cancer cells show reduced proliferation in conditions under which TGFβ2 levels are high. Additionally we could now show that bone resident macrophages are the direct source of TGFβ2, indicating that bone macrophages induce dormancy in disseminated cancer cell reaching the bone microenvironment. Lung macrophages do not express high levels of TGFβ2 and seem to further inhibit the production of TGFβ2 in the lung microenvironment.

As proposed in SA2.2b we are currently pursuing a non-targeted approach to identify unknown MΦ-derived factors that mediate induction and exit from dormancy. To this end, we are analyzing conditioned medium as it was used for the TGFβ2 ELISA by mass spectrometry to detect proteins that are a) secreted by BM MΦs and induce cancer cell dormancy or b) secreted by L MΦs and allow escape from dormancy. The results of the mass spectrometry analysis are not available yet. This effort will be continued in the lab of the PI's mentor Dr. Aguirre-Ghiso.

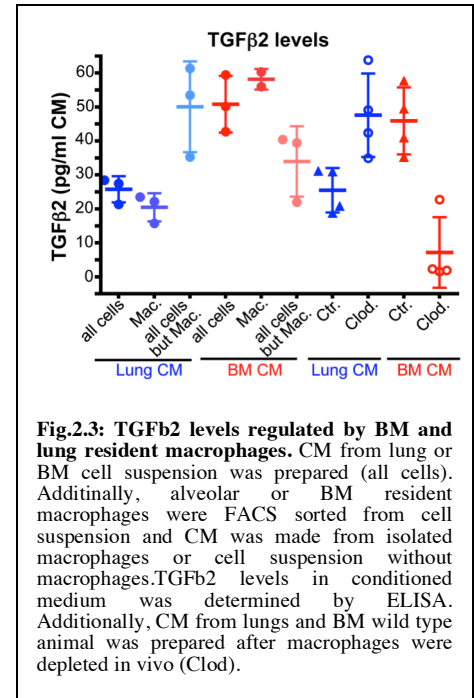
Task 3: Validation of MΦ-derived dormancy inducing factors.

This effort was proposed for funding year 3 in SA2.3.

Training opportunities. The training opportunities according to the statement of work were met; these training opportunities were: sorting of mammary gland macrophages; performing RealTime PCRs, performing 2D and 3D in vitro cultures, use combinatory pharmacologic inhibitors, isolate and cultivate BM and lung MΦs, perform mammosphere cultures, perform tail vein injections, perform cryosections. Additionally, meetings with the Co-mentor Dr. Merad and Dr. Aguirre-Ghiso were held as well as with the mentoring committee to discuss progress of the project.

Dissemination of results to the community. The work presented here is part of a research manuscript that is under revision in Nature Communications. Additionally, the results have been presented in several conferences (see Product section).

Plans for the next funding period. The PI has accepted a lab head position with Merck KGaA in Germany and will therefore not be able to continue the work in the next funding period. The PI's mentor will most likely continue the project and apply for additional funding sources to do so.



5. IMPACT

Approximately 90% of breast cancer patients die from commonly incurable metastases. Metastases can occur years or decades after removal of the primary tumor, suggesting there is a window of opportunity to prevent their outgrowth. Additionally, recent clinical data demonstrating that dissemination can occur much earlier than assumed and that patients carrying pathologically defined pre-invasive breast cancer lesions (e.g. ductal carcinoma in situ, DCIS) can carry dormant DCCs. This has caused great confusion on how to treat women with early stage breast cancer such as DCIS. A recent study published in JAMA Oncology [10] adds to this confusion as it shows that while breast cancer deaths from DCIS are rare, 50% of those cases occur in the absence of an invasive breast cancer recurrence and that the choice of therapy did not affect survival. This indicates that albeit at early frequency, a subpopulation of women with DCIS carry early disseminated cancer cells that can have deadly consequences. We therefore need better tools to identify those DCIS patients at high risk of developing late metastatic relapses without overtreating the majority of women who have harmless variants of DCIS. Our mechanistic work on early dissemination that we were able to carry out with the support of this DoD breast cancer award has led us to the notion that those DCIS lesions carrying an E-Cadherin low/macrophage high signature might be indicative of the presence of disseminated disease.

6. CHANGES/PROBLEMS

No major changes or problems.

7. PRODUCTS

Manuscripts:

„Macrophages orchestrate early dissemination of HER2+ cancer cells.“ Nina Linde, Arthur Mortha, Eduardo Farias, Maria Soledad Sosa, Kathryn Harper, Ethan Tardio, Miriam Merad, and Julio A. Aguirre-Ghiso. – *Manuscript under revision in Nature Communications.*

„The relationship between dormant cancer cells and their microenvironment.“ Nina Linde^{*#}, Georg Fluegen^{*#}, and Julio A. Aguirre-Ghiso[#]. – *Invited Book Chapter for **Advances in Cancer Research**. In press. *Both authors contributed equally. #Corresponding author.*

Oral Presentations:

„Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells“
Cancer Biology Retreat, Mount Sinai, New York, NY, December 2014

Awards:

Icahn School of Medicine at Mount Sinai Postdoctoral Recognition Award, New York, December 2014
AACR Scholar in Training Award 2016

Poster presentations:

„Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells“
Annual Meeting of the American Society of Cell Biology, Philadelphia, PA, December 2014

„Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells“
Cancer Cell Symposium on Cancer Inflammation, Sitges, Spain, June 2015

„Macrophages orchestrate early dissemination of HER2+ cancer cells“ Annual Meeting American Association of Cancer Research, New Orleans, LA, April 2016

„Macrophages orchestrate early dissemination of HER2+ cancer cells“ American Association of Cancer Research Tumor Metastasis Meeting, Austin, TX, November 2015

8. PARTICIPANTS

no change

9. LITERATURE

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10. APPENDIX: Manuscript on the following pages

Macrophages orchestrate early dissemination and metastasis of HER2+ cancer cells.

Nina Linde¹, Arthur Mortha², Adeeb Rahman³, Eduardo Farias¹, Maria Soledad Sosa¹, Kathryn Harper¹, Ethan Tardio¹, Miriam Merad^{2,3}, and Julio A. Aguirre-Ghiso^{1,4}.

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Abstract

Cancer cell dissemination can occur during very early stages of breast cancer but the mechanisms controlling this process and how they contribute to metastasis are unclear. Here we show that MMTV-HER2 early cancer lesions contain an invasive subpopulation of HER2⁺/E-cadherin^{lo} cancer cells that depend on macrophages for dissemination. Macrophages produced Wnt-1 and induced loss of E-cadherin and dissemination of early HER2⁺ cancer cells. Depletion of macrophages before overt tumor detection drastically reduced early dissemination and diminished the onset of metastasis even when macrophage depletion was stopped when tumors became invasive. Resident CCR2⁺/CD206⁺/VCAM-1⁺/Tie2⁺ macrophages were attracted into early lesions by CCL2 produced by early HER2⁺ cancer cells in an NFκB-dependent manner. Intra-epithelial macrophages and loss of E-cadherin junctions was also found in human DCIS, but not normal breast tissue. We reveal a previously unrecognized mechanism by which macrophages play a causal role in early dissemination impacting long-term metastasis development.

Introduction

The paradigm of cancer metastasis states that dissemination and metastasis occur when advanced aggressive tumors acquire invasive mechanisms. The finding that dissemination does not only occur from late stage invasive tumors has challenged this model ¹. Large cohort patient studies ²⁻⁵ and studies with spontaneous mouse tumor models ⁶ showed that dissemination also occurs during early stages of cancer when lesions are diagnosed by light microscopy as pre-malignant or pre-invasive. In addition, cancer of unknown primary is a relatively frequent event in solid cancers where metastases develop without the presence of an obvious primary tumor mass that evolved to become invasive ⁷.

The “early dissemination” definition was refined by Husemann et al. ⁶ when they showed that early disseminated cancer cells (DCCs) originate at times when lesions are only defined *in situ* by light microscopy (e.g. ductal carcinoma in situ (DCIS) in humans and mammary intraepithelial neoplasia in mice) but dissemination occurs and early DCCs show few genetic aberrations. In the MMTV-HER2 model, early DCCs are able to form lung metastases in the absence of invasive carcinoma ⁶. This argues that in these models early DCCs are endowed with latent metastasis initiating capacity. Similarly, women treated for DCIS can develop metastases without ever developing any subsequent local invasive breast cancer ⁸⁻¹². This might indicate that, albeit at low frequency, early DCCs can unpredictably form metastases in patients. Early dissemination is not a rarity of breast cancer models (MMTV-HER2 and -PyMT models ⁶, as it also occurs in spontaneous mouse models of melanoma ¹³ and pancreatic cancer ¹⁴.

While in K-Ras-driven pancreatic cancer an EMT has been linked to early dissemination¹⁴, its contribution to metastasis is unknown. Further, it remains poorly understood how dissemination occurs during pre-invasive stages of breast cancer when the epithelium-to-stroma barrier is intact. Early DCCs displayed only few genetic alterations^{4,6}, indicating that early dissemination might be driven by epigenetic and micro-environmental mechanisms that turn on programs of epithelial cell motility^{15,16}. In fact, invasion of epithelial cells occurs physiologically during development.

The mammary epithelium forms postnatally during adolescence in a process called branching morphogenesis where rapidly dividing epithelial cells elongate the terminal end bud into the fat pad and bifurcate into the ductal tree. Macrophages are key regulators of branching morphogenesis during mammary gland development^{17,18}, arguing that normal mammary epithelial cells cooperate with these innate immune cells for invasive processes. These data led to the discovery of macrophages as powerful drivers of intravasation from invasive breast cancer tumors *via* the establishment of tumor microenvironments of metastasis¹⁹. This follows a streaming process where breast cancer cells recruit macrophages *via* production of colony stimulating factor 1 (CSF1) and then cancer cell motility is stimulated *via* macrophage-derived EGF²⁰. Additionally, macrophages can induce an epithelial to mesenchymal transition (EMT) in malignant cells^{21,22}. However, the role of macrophages in early spread of cancer remained unexplored.

Here we show that the branching morphogenesis program is altered by the HER2 oncogene early in cancer development. Mammary tissue macrophages are recruited by HER2+ early cancer cells from the stroma into the epithelial layer of lesions

defined as mammary intraepithelial neoplasia in mice (similar to DCIS in humans)²³. This process depends on HER2-NFkB-mediated induction of CCL2. CCR2+ intra-epithelial macrophages respond to CCL2, produce Wnt-1 and disrupt the myo-epithelial layer locally by downregulating E-cadherin junctions in a subpopulation of HER2+ cancer cells. Before tumors form, these events result in early dissemination microenvironments that propel active intravasation and dissemination to the lung that was efficiently blocked by macrophage depletion. Transient depletion of macrophages in mice before the formation of invasive tumors reduced lung metastatic burden. Importantly, this was the case when macrophage depletion had been stopped with formation of invasive tumors and mice were followed until they carried large overt invasive tumors. Our results suggest a previously unrecognized role for HER2-mediated recruitment of macrophages to favor dissemination of HER2+ tumor cells much earlier than growth is induced by the oncogene. Our work also reveals a role for early DCCs in supporting late metastasis development.

RESULTS

MACROPHAGES INFILTRATE THE EPITHELIAL COMPARTMENT OF EARLY HER2+ MAMMARY CANCER LESIONS.

We asked whether the HER2 oncogene might recruit macrophages to orchestrate early dissemination. We used MMTV-HER2 mice as a murine model of breast cancer since these show slow progression from early lesions such as hyperplasia and mammary intra-epithelial neoplasia (**Fig.1A,B**), the latter a similar lesion to DCIS²³, to invasive tumors (**Fig.1C**). We stained MMTV-HER2 mammary

gland sections for the murine macrophage marker F4/80 before we could detect any signs of invasive tumor masses in serial sections of mammary tissue or even enhanced proliferation in HER2+ early lesions²⁴. Macrophages were located to the stroma outside of mammary ducts in healthy FVB wild type animals (**Fig.1D**). This was also true in young 14wk old MMTV-HER2 mice (**Fig.1E**). However, when MMTV-HER2 mice progressed over time but still showed no signs of tumor masses or enhanced proliferation²⁴, macrophages were often localized inside the luminal epithelial layer of early lesions as demonstrated by co-staining of F4/80 and cytokeratin 8/18 (**Fig. 1F**). We hypothesized that as macrophages enter the early lesions, they might disrupt the architecture of the duct. Close inspection of sections co-stained for smooth muscle actin and F4/80 showed that the myoepithelial cell layer was frequently disrupted at sites where macrophages were in immediate contact with the duct (**Fig. 1G-I**). Quantification of the abundance of intra-epithelial macrophages confirmed that the incidence of ducts with intra-epithelial macrophages correlated with HER2 upregulation and disease progression (**Fig. 1J**).

MACROPHAGES DISMANTLE E-CADHERIN JUNCTIONS IN EARLY HER2+ CANCER CELLS.

We hypothesized that HER2 might aberrantly activate a mechanism of invasion and motility involving macrophages in early lesions. We found that intra-epithelial macrophages were associated with a strong local downregulation of E-cadherin junctions *in vivo* in early lesion cells located directly adjacent to macrophages (1-2 cell diameter away) (**Fig.2A-C**). This was paralleled by a general downregulation of E-Cadherin mRNA in early lesions compared to wild type glands (**Fig.2D**). Additionally, β -

Catenin levels were increased in early lesions containing intra-epithelial macrophages as measured by dual color IHC (**Fig.2E-G**). A loss of E-Cadherin and translocation of β -Catenin to the nucleus could also be induced *in vitro* when Raw264.7 macrophages were added to Comma-1D healthy mammary epithelial cell monolayers used as a readout for epithelial junction formation (**Fig.2H-J**). The loss of E-cadherin junctions in epithelial cells adjacent to macrophages suggested that macrophages might produce cues that stimulate a loss of E-cadherin junctions as observed during the epithelium to mesenchyme transition (EMT). Macrophages can produce Wnt ligands²⁵⁻²⁷ which are potent inducers of an EMT. We therefore tested the response of Raw264.7 macrophages or primary mammary tissue macrophages isolated from early lesions to conditioned media from healthy epithelial cells or from HER2+ early cancer cells. Only conditioned media derived from HER2+ cells induced an upregulation of Wnt-1 (**Fig.2K, L**); no changes were detected for Wnt-3, Wnt5a and Wnt7 (data not shown). The loss of E-Cadherin junctions in Comma-1D cells induced by Raw264.7 macrophages was reversed by addition of DKK1, an inhibitor of canonical Wnt signaling, to the co-cultures (**Fig.2M-P**). We conclude that downregulation of E-cadherin mRNA and junctions and β -catenin upregulation and nuclear translocation in early lesion cells results from HER2-dependent recruitment of Wnt-1 secreting macrophages into the early lesions.

MACROPHAGE DEPLETION PREVENTS AN E-CADHERIN AND β -CATENIN JUNCTION DISASSEMBLY AND EARLY DISSEMINATION.

Our data indicated that HER2 leads to macrophage mobilization into early lesions where they induce an EMT in early cancer cells. We next tested whether this process leads to early dissemination. CSF1R is expressed by most tissue resident macrophages

and is required for macrophages survival in tissues²⁸. Thus, we injected MMTV-HER2 mice during early lesions with a blocking antibody to Csf-1 receptor (CSF1R) to eliminate macrophages from early lesions and then quantified circulating and disseminated early cancer cells in blood and target organs respectively (**Fig.3A**). CSF1R blockade led to efficient depletion of tissue resident CD11b⁺/F4/80⁺/Gr1⁺ macrophages (**Supplementary Fig.1A**). Immunofluorescence staining for F4/80 in HER2⁺ early lesions confirmed a significant reduction in the number of intra-epithelial macrophages when CSF1R was blocked (**Supplementary Fig.1B**). We confirmed that at the end of the experiment, no tumor masses had formed, by inspecting whole mount stainings of mammary glands (**Supplementary Fig.1D,E**) and HE staining of serially sectioned mammary tissue (**Fig.3B,C**). Macrophage depletion was accompanied by a significant reduction in the number of hyperplastic ducts (**Supplementary Fig.1C**) and a tissue wide upregulation of E-Cadherin mRNA (**Fig.3D**) and E-Cadherin-based junctions (**Fig.3E-G**). We conclude that macrophages contribute to the loss of E-cadherin mRNA and junctions and disrupted mammary tissue architecture in early lesions.

The above changes correlated with the finding that CSF1R blockade significantly reduced the numbers of early circulating cancer cells (**Fig.3H**). Accordingly, CSF1R blockade also reduced early disseminated cancer cell (DCC) burden in target organs as measured by the detection of the transgene surface HER2 expressing early DCCs in the lungs (**Fig.3I-K**). We conclude that macrophages play a critical role in the ability of early cancer cells to acquire an invasive and disseminating phenotype. As indicated previously, these events of dissemination take place during a stage where no invasive

tumors are detectable and early lesions show similar proliferative capacity than normal resting mammary epithelium²⁴.

HER2-RECRUITED MACROPHAGES CONTROL EARLY DISSEMINATION AND CONTRIBUTE TO METACHRONOUS METASTASIS FORMATION.

We next tested whether macrophage-regulated early dissemination contributed to metastasis formation. To this end we blocked CSF1R only during early asymptomatic stages of cancer, starting at age week 18, and stopped as soon as tumors became palpable (size <3mm in diameter). We then waited until tumors reached 1 cm in diameter (4-6 weeks later) and quantified solitary DCCs and metastatic lesions in lungs (**Fig.4A**). We found that the time to tumor detection was slightly delayed when macrophages were depleted during asymptomatic pre-malignant stages (**Fig.4B**). However, once palpable tumors had formed, the progression to overt tumors was not affected (**Fig.4C**). Additionally, overt tumors showed no difference in macrophage content (**Fig.4D-F**) and vascularization (**Fig.4G-J**) at the end of the experiment between control and anti-CSF1R treated mice. This suggests that there is no impact on overt tumor growth in late tumors when macrophages were depleted during early stages. Additionally, flow analysis of lungs revealed that neither alveolar macrophage nor CD11b+/Gr1+ monocyte content was significantly affected (**Supplementary Fig.2A-E**) by the same treatment arguing against a systemic loss of all macrophages. However, CSF1R blockade during early stages significantly decreased solitary DCC burden in lungs (**Fig.4K**). CSF1R blockade during early stages also caused a statistically significant decrease in the number of metachronous metastases per mouse (**Fig.4M**),

which were defined as the number of metastatic lesions bigger than three cells (**Fig.4L**). This inhibitory effect of DCC burden and metastasis was detected even after macrophage depletion had been stopped in average for one month and animals had carried fast-growing tumors. That the number of solitary DCCs, likely a mixture of DCCs accumulated since the early stages was reduced by CSF1R blockade suggests that the reduced influx of DCCs to lungs during early stages was not replaced by DCCs arriving during the time of tumor detection to euthanasia when tumors were large. We conclude that HER2+ early cancer cells recruit macrophages and that these play a seminal role in early dissemination, allowing for the early DCCs to reach target organs and form metastasis.

HETEROGENEITY OF MYELOID CELLS IN EARLY MIN LESIONS.

Our data suggests that macrophages play an active role in favoring early dissemination and that early DCCs contribute to metachronous metastasis formation. Remarkably, this occurs during early cancer stages in the absence of highly proliferative tumor masses when the mammary tree mostly resembles that of a healthy gland. We hypothesized that dissemination might be facilitated by tissue resident macrophages involved in programs of branching morphogenesis during mammary gland development^{17,29}. We therefore compared the phenotype of macrophages present in wild type and early lesion mammary glands. Mammary glands derived from FVB wild type mice and MMTV-HER2 early lesion at 14 and 22 weeks were analyzed using CyTOF with a panel of 17 hematopoietic cell markers and IdU as a proliferation marker (**Supplementary Fig.3A**). Myelo-monocytic cells were identified as CD45+CD11b+F4/80+ cells lacking

lymphoid and granulocytic markers (**Supplementary Fig.3B**). viSNE plots³⁰ of myelomonocytic cells (**Fig.5A**) showed that myelomonocytic numbers and patterns were similar between wild type glands and early lesions. viSNE analysis further revealed three putative populations, one of which could be identified as monocyte based on its high Ly6C levels (**Fig.5B**) and did not differ in numbers between these samples (**Fig.5D**). The remaining LY6C⁻ macrophages could be distinguished into two populations according to the expression of CD206 (**Fig.5C**). The pre-dominant macrophage subtype present in both wild type glands and early lesions was CD206^{hi} (**Fig.5E,F**). CD206^{hi} macrophages were also Tie2^{hi}, CD11b^{hi} and VCAM^{lo} and did not show any signs of proliferation based on IdU incorporation (**Fig.5I,J; Supplementary Fig.3C,D**). This matches previous findings³¹ that wild type resident mammary glands contained M2 polarized CD11b^{hi}, CD206^{hi} and Tie2^{hi} macrophages termed mammary tissue macrophages (MTMs) whereas tumor-associated macrophages prevalent in invasive MMTV-PyMT tumors were CD11b^{int}, CD206^{lo}, Tie2^{lo} and VCAM^{hi}. We found that these CD206^{lo} TAMs were a minority in wild type glands or early lesions but indeed constituted the majority of myelomonocytic cells in overt invasive MMTV-HER2 tumors (**Fig.5G,H**). Since our global CyTOF analysis showed that the predominant macrophage population in HER2⁺ early lesions resembled MTMs, we wanted to correlate the macrophage status with their localization and performed *in situ* stainings to identify whether intra-epithelial macrophages in early lesions might be CD206^{hi} MTMs as well. To this end we co-stained wild type glands, early lesions and overt tumors tissues against F4/80 and CD206 (**Fig.5K-M**). We found that stromal and intra-epithelial macrophages in both wild type glands and early lesions were CD206^{hi} whereas tumor

associated macrophages in overt tumors were CD206^{lo} (**Fig.5N**). While tracing the exact origin and lineage of macrophage subtypes will require further scrutiny, our data suggest that intra-epithelial macrophages might be derived from resident mammary tissue macrophages and that expansion of monocyte derived tumor associated macrophages is a hallmark of overt tumor stages.

HER2+ EARLY CANCER CELLS PRODUCE CCL2 AND RECRUIT MACROPHAGES INTO MAMMARY DUCTS.

We next explored what signals might HER2 upregulation induce that might attract resident mammary tissue macrophages from the stroma into the ductal epithelium of HER2+ early lesions. In invasive breast cancer models, HER2 signaling activates NFκB, which transcriptionally induces CCL2, a potent macrophage chemotactic factor ³². Accordingly, the p65 subunit of NFκB subunit was phosphorylated in mammospheres derived from HER2+ early cancer cells, and Lapatinib, a HER2 and EGFR inhibitor, inhibited its phosphorylation (**Fig.6A**). We then isolated RNA from mammospheres derived from either FVB wild type or MMTV-HER2 early MIN lesions as described ³³ and performed quantitative real-time PCR analysis for cytokine mRNAs. We found that already at these early stages of progression, when enhanced proliferation is not yet detectable ²⁴, HER2+ cancer cells upregulated expression of CCL2 but not CSF2, CSF1, IL1β, and IL6 (**Fig. 6B, Supplementary Fig.4A,B**). Upregulation of CCL2 around HER2+ early cancer cells was also observed at the protein levels as revealed by immunofluorescence (**Fig.6C,D, Supplementary Fig.4C-E**). CCR2+ cells could be found close to CCL2+ early cancer cells (**Fig.6C insert**). Additionally, acinar-like

265 structures produced by early MMTV-HER2 cancer cells displayed a reduction in
266 secreted and peri-organoid CCL2 production upon inhibition of HER2 or NFkB signaling
267 with specific inhibitors ³⁴ (**Fig.6E-G**). To confirm that CCL2 signaling was necessary and
268 sufficient for HER2-dependent macrophage recruitment, HER2+ early cancer cells were
269 grown as 3D acinar structures *in vitro* for 5 days. Cultures were then treated with
270 Lapatinib, an IKK β inhibitor ³⁴ or an inhibitor of the CCL2 receptor CCR2 (RS504393)
271 and macrophages isolated from mammary glands of MMTV-HER2 mice were added to
272 the cultures. After 24 hours of co-culture, macrophages were associated with ~50% of
273 all organoid structures in control samples (**Fig.6H**). In contrast, co-cultures treated with
274 the inhibitors all showed significant reduction in macrophage association (**Fig.6I,J**). We
275 next treated 20 week old MMTV-HER2 mice carrying only early lesions (no palpable or
276 overt tumors) for 2 weeks with a CCR2 inhibitor (**Fig.6K,L**). We found that the number
277 of intra-epithelial macrophages was significantly reduced when mice were treated
278 systemically (**Fig.6M**). When the CCR2 inhibitor was administered locally into the fat
279 pad to avoid systemic effects (**Fig.6N**), intra-epithelial macrophage content was reduced
280 compared to contra-lateral control treated glands (**Fig.6O**), supporting that intra-
281 epithelial macrophages are indeed derived from resident mammary gland macrophages
282 instead of circulating monocyte derived macrophages. Additionally, mammary tissue
283 macrophages can also be depleted by genetic ablation of CCR2 ³¹ arguing that we may
284 be eliminating the same population during early mammary cancer stages. We conclude
285 that HER2 signaling in cancer cells from early lesions activates NFkB to induce CCL2,
286 which in turn mobilizes CCR2+ resident mammary macrophages from the stroma into
287 these early lesions.

HIGH INTRA-EPITHELIAL MACROPHAGE NUMBERS IN PATIENT DCIS LESIONS CORRELATE WITH LOW E-CADHERIN LEVELS.

Published data showed that more than 10% of patients with DCIS had detectable DCCs in their bone marrow (BM), but no histologic markers, which include invasive fronts and receptor status were indicative of the presence of DCCs³. To test whether macrophages could also infiltrate early lesions in humans, we compared macrophages in healthy human breast tissue vs. tissue from DCIS lesions as a model of early stage breast cancer lesions. Macrophages were identified as CD68+/CD45+ and cytokeratin8/18 negative cells (**Supplementary Fig.5A,B**). In breast tissue from healthy donors, macrophages were localized in the stroma in the vicinity of mammary ducts but remained outside of the ducts, which were delimited by an intact myoepithelial layer of cells (**Fig.7A**). In contrast, even in DCIS lesions that displayed an apparently intact myoepithelial layer, there was a statistically significant increase in the frequency of macrophages found inside the aberrant ductal epithelial structure in between cancer cells (**Fig.7B,C**). The association of intra-epithelial macrophages with reduced E-Cadherin levels was confirmed in human DCIS samples (N=12). This was independent of HER2 status and appeared in different patterns. Patients with high macrophage numbers within lesions showed overall lower E-Cadherin levels as measured by quantitative image analysis (**Fig.7D-F**). Additionally, within the same patient, individual lesions with high macrophage numbers had lower E-Cadherin levels (**Supplementary Fig.5C-E**). This reveals an inter- and intra-tumor heterogeneity and suggests that some regions of DCIS lesions might be more prone to contain cancer cells able to undergo early dissemination.

Discussion

Following the assumption that dissemination occurs only during late invasive cancer stages, many studies have focused on investigating dissemination from invasive lesions. However, micro-invasion events were detected in patients' "pre-invasive" DCIS lesions using electron microscopy, but these events might go unnoticed by light microscopy⁶. Additionally, DCIS lesions were found to be vascularized³⁵, indicating that there might be a possibility for early cancer cell dissemination within the DCIS lesions. Our data reveal that macrophages enter the epithelium of early lesions in mice and human DCIS where they create early dissemination microenvironments. In mice this process can be initiated by the HER2 oncogene before growth stimulation is evident, revealing a novel function for the HER2 oncogene that could be targeted. The HER2 orchestrated early dissemination microenvironments contain early cancer cells that attract CCR2+ macrophages *via* CCL2 secretion that in turn secrete Wnt-1 to dismantle epithelial E-Cadherin junctions. The dissemination-promoting function of macrophages was proven when we found that depletion of macrophages in early HER2+ lesions using anti-CSF1R antibodies reversed the loss of E-cadherin in HER2+ lesions as well as intravasation and dissemination to lungs. Interestingly, Wnt signaling is linked to branching morphogenesis^{36,37} and a subset of tumor-associated macrophages that drive invasive cancer dissemination also secrete Wnt ligands²⁷.

We found that the myelo-monocytic landscape of HER2+ early lesions at the time of early dissemination resembled that of healthy mammary glands and largely consisted of M2 polarized F4/80+/CD11b+/CD206+/Tie2+ macrophages. In contrast, overt tumors predominantly contained CD11b^{int}/CD206^{lo}/Tie2^{lo}/VCAM+ macrophages as described³¹

but these were the minority of macrophages in wild type and early lesion ducts. We corroborated these findings *in situ* by immunofluorescence staining, confirming that intra-epithelial macrophages and wild type resident macrophages are CD206⁺ whereas tumor associated macrophages are CD206^{lo}. Interestingly, the profile of resident and early lesion macrophages is characteristic for a subset of macrophages in invasive breast cancer lesions that are gatekeepers of intravasation doorways ¹⁹.

This raises the possibility that when HER2 is overexpressed in mammary epithelial cells, resident macrophages have the inherent potential to aberrantly fuel epithelial cell motility as is the case during physiologic mammary gland development ^{17,18,29}. While the barrier between stroma and epithelium prevents resident macrophages from disrupting the steady state epithelial architecture, oncogene-activated attraction of resident macrophages into the epithelium might result in disruption of normal tissue boundaries and early dissemination. However, further scrutiny and lineage tracing experiments are required to fully understand the origin of macrophages driving early and late dissemination.

We further found that when macrophages were depleted during early stages but allowed to rebound during invasive stages, lung metastatic burden was still reduced. This indicates that early DCCs contribute to lung metastasis formation, either directly or indirectly and surprisingly large tumors that persisted in mice for ~1.5 months were not able to compensate for the reduced dissemination during early stages. While the exact clinical implications of our findings need further analysis, a few scenarios of clinical relevance can be discussed. It is argued that because 13% of DCIS patients show disseminated disease and only 3% develop metastatic disease ⁸⁻¹², early dissemination

is not a contributor to lethal metastatic cancer. However, approximately 50% of breast cancer deaths after DCIS occurred in the absence of a detectable local invasive disease and were not influenced by current treatments¹⁰. Further, cancer can manifest with metastasis without a detectable primary even after careful inspection of the patients³⁸. These clinical findings indicate that at least in a subgroup of patients, early DCCs may develop lethal metastases¹⁰ (**Fig.7G** scenario 1). Additionally, early DCCs may cooperate with later arriving DCCs to form metastasis in patients that after DCIS treatment go on to develop invasive lesions or in patients that had DCIS but only were diagnosed later for invasive cancer (**Fig.7G** scenario 2). This resembles the concept of the “pre-metastatic” niche described previously^{39,40} where the pre-metastatic niche could also be orchestrated by early DCCs. Next phase studies will address the genetic identity of metastasis affected by macrophage depletion and determine the contribution of early DCCs to the metastatic burden late in cancer progression.

Overall, our studies suggest that before propelling rapid growth, oncogenes such as HER2 might turn on developmental programs of anoikis resistance²⁴, macrophage recruitment and invasion that initiate dissemination much earlier than anticipated. We provide critical new insight into the understanding of the natural history of metastatic disease and we demonstrate that macrophages and early DCCs appear to play a seminal role in metastatic breast cancer.

Methods.

Cells and cell culture. Raw264.7 cells expressing mCherry were generated using mCherry lentiviral vectors and maintained in DMEM (Lonza) with 10% FBS and 1%

Pen/Strep. Comma-1D cells were maintained in DMEM-F12 medium containing 2%FBS, 1% Pen/Strep. For DKK1 stimulation, conditioned media was prepared from DKK1 expressing 293T cells and protein concentration was determined using a Bradford assay cells cultured with serum free medium (DMEM + 1%P/S) for 24 hours and then concentrated using Vivaspin 20 Centrifugal Concentrating tubes (Sartorius, VS2021) at 3000g up to 3 hours until desired concentration (10x) was reached. 0.5ug/ml DKK1 protein was used for stimulation. For co-culture experiments, Comma-1D cells were seeded on coverslips and after 12h, Raw-264.7-mCherry cells were added. Co-cultures were fixed in 2% formalin after 12h and then stained. All cell lines were routinely tested for mycoplasma.

Mouse experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at Mount Sinai, protocols 08-0366 and 2014-0190. FVB/N-Tg(MMTVneu)202Mul/J or FVB wild type mice were purchased from Jackson laboratory and bred in-house. Animals were sacrificed using CO₂ at age 14, 20-22wk or when invasive tumors had reached a diameter of 1cm. For macrophage depletion, we administered 3mg of the CSF1R antibody clone ASF98 on day 1 and 1mg on day 7 and weekly thereafter by injection into the tail vein of 18wk old pre-malignant MMTV-Her2 mice. PBS was used as vehicle control. Treatment lasted 14 days or until tumors were first palpable (3mm diameter). ASF98 antibody was a generous gift of Dr. Miriam Merad. For CCR2 blockade, mice were either treated with 2mg/kg of RS504393 (Tocris Bioscience) or

402 vehicle control (DMSO) daily by i.p. injections for 14 days or by injection of 1mg/kg
403 RS504393 into the fat pad for 5d.

404 **Mammary Gland Whole Mounts.** Mammary glands were excised, fixed in Carnoy's
405 fixative and stained in carmine alum solution as described
406 in <http://mammary.nih.gov/tools/histological/Histology/index.html>.

407 **Microscopy.** Formalin fixed and paraffin embedded samples were prepared and
408 stained as described ⁴¹. For immunohistochemistry, VectaStain Elite ABC Rabbit IgG
409 and Mouse IgG (PK-6102) kits from Vector Laboratories were used for secondary
410 antibodies. Secondary antibodies were left for one hour at room temperature. DAB and
411 Vector Blue substrate kit (Vector Laboratories) were used for enzymatic substrate.
412 Mounting was done using VECTASHIELD mounting media (Vector Laboratories). For
413 CD206 stainings, cryosections were used. Tissue was fixed in 4% formalin over night,
414 incubated in 30% sucrose/PBS over night and sectioned into 6mm sections. Staining of
415 cryosection was done as described ⁴². Antibodies used were: CD68 (Sigma, polyclonal),
416 F4/80 (abcam CIA:3-1), Iba-1 (Wako polyclonal), Cytokeratin 8/18 (Progen, polyclonal),
417 smooth muscle actin (Sigma IA4), CD206 (Biolegend C068C2), CCL2 (Novus 2D8), E-
418 Cadherin (Becton Dickinson, polyclonal), beta catenin (cell signaling, polyclonal), Her2
419 (abcam, polyclonal), Endomucin (Santa Cruz 7C7). For costaining of F4/80 and CD206
420 (both raised in rat) a FITC-conjugated F4/80 antibody (Biolegend BM8) was used in
421 combination with CD206 (Biolegend C068C2) in a sequential stain. Microscopic
422 analysis was carried out with a Leica widefield microscope or with a Leica confocal
423 microscope for 3D cultures. For quantification of immunofluorescence signal intensity
424 with the Metamorph software, regions of interest were defined in original tiff files that

had been taken under the same exposure time and settings and the mean signal intensity was measured. Because the use of a directly conjugated F4/80 antibody resulted in lower signal intensity, we used Iba1 as a macrophage marker ¹⁹ to identify macrophages for CD206 signal intensity measurement instead.

Flow Cytometry. MMTV-HER2 mice were sacrificed using CO₂ at age 18-22wk (early pre-malignant cancer lesions) or when overt tumors had formed. Whole mammary glands or tumors were digested in Collagenase/BSA at 37°C for 30-45min. Red blood cell lysis buffer (Sigma) was used to remove blood cells. Cell suspensions were blocked with Fc-blocking reagent (eBioscience) and samples were surface stained in FACS buffer (PBS supplemented with 1% BSA and 2mM EDTA) for 20-30 min on ice using the following antibodies: CD45-PerCPCy5.5 (Biolegend 30-F11), CD11b-PeCy7 (eBioscience M1/70), CD11c-PE (eBioscience N418), Gr1-AF700 (eBioscience RB68C5), Tie2-biotin (eBioscience TEK4), F4/80-biotin (Biolegend BM8), CD206-APC (Biolegend C068C2), VCAM-FITC (eBioscience 429). DAPI was used to label dead cells. Multiparameter analysis was performed on a Fortessa (BD) and analyzed with FlowJo software (Tree Star). DAPI+ cells and doublets were excluded from all analysis. To sort mammary tissue macrophages, whole mammary glands from 14wk, 18-22wk (early pre-malignant cancer lesions) mammary glands or from invasive tumors were digested in Collagenase/BSA at 37°C for 30-45min. Mononuclear cells were enriched in a Percoll gradient and then macrophages were sorted as viable CD45+/Gr1-/CD11b+/F4/80+ cells.

CyTOF analysis. All mass cytometry reagents were purchased from Fluidigm Inc. (former DVS) unless otherwise noted. Mice were injected i.p. with 1mg IdU per mouse 16h prior to the experiment. Lymph nodes were removed and mammary glands were digested using the Miltenyi fatty tissue digestion kit. Cells were then washed with PBS containing 1% BSA and blocked with Fc-blocking reagent (eBioscience) to minimize non-specific antibody binding. Cells were stained with a panel of metal-labeled antibodies against 20 cell surface markers (Fig.5 supplement 1A) for 30 minutes on ice, and then washed. All antibodies were either purchased pre-conjugated to metal tags, or conjugated in-house using MaxPar X8 conjugation kits according to the manufacturer's instructions. After antibody staining, cells were incubated with cisplatin for 5 minutes at RT as a viability dye for dead cell exclusion. Cells were then fixed and permeabilized with a commercial fix/perm buffer (BD Biosciences) and stored in PBS containing 1.6% formaldehyde and a 1:4000 dilution of Ir nucleic acid intercalator to label all nucleated cells. Immediately prior to acquisition, cells were washed in PBS, and diH2O and resuspended in diH2O containing a 1/10 dilution of EQ 4 Element Calibration beads. After routine instrument tuning and optimization, the samples were acquired on a CyTOF2 Mass Cytometer in sequential 10min acquisitions at an acquisition rate of <500 events/s. The resulting FCS files were concatenated and normalized using a bead-based normalization algorithm in the CyTOF acquisition software and analyzed with Cytobank. FCS files were manually pre-gated on Ir193 DNA+ CD45+ events, excluding dead cells, doublets and DNA-negative debris. Myeloid derived cells were manually gated based on CD11c and CD11b expression and the gated myeloid populations were then analyzed using viSNE³⁰ based on all myeloid phenotypic markers. Putative cell

populations on the resulting viSNE map were manually gated based on the expression of canonical markers, while allowing for visualization of additional heterogeneity within and outside of the labeled population bubbles.

Mammospheres and 3D mammary primary epithelial cell cultures. Acini cultures were performed as described^{33,43}. 5×10^4 eCCs were seeded in 400ul Assay Medium in 8-well chamber slides coated with 40ul of Matrigel (Corning). Acini formed at an efficiency of around 30 acini/ 1×10^4 MECs plated. For macrophage co-cultures, primary tissue macrophages were added at a ratio of 500 per 1×10^4 eCCs seeded to 5d old acini cultures. For inhibitory treatments, 5d old acini cultures were treated for 24h with 1 μ M Lapatinib (LC Laboratories), 2 μ M IKK Inhibitor³⁴ (generous gift from Dr. Albert Baldwin), 1 μ M CCR2 inhibitor RS504393 (Tocris Bioscience) or DMSO as vehicle control. Cultures were then fixed for immunofluorescence (IF) with 4% PFA. Mammosphere cultures were prepared as described³³. To prepare conditioned medium, 5d old mammosphere culture were plated in serum free DMEM medium and conditioned medium was harvested after 24h.

Immunoblotting, RT-PCR, and quantitative PCR (qPCR). Immunoblotting was performed as described previously^{44,45}. Antibodies used were P-NF-kappa-B p65 (Cell Signaling polyclonal) and beta-tubulin (abcam, polyclonal). For expression analysis of FVB wild type mammary epithelial cells or MMTV-Her2 eCCs, epithelial cells were isolated and grown as mammospheres for 5d as described³³. For expression analysis of Raw264.7 macrophages, cells were grown as monolayers in 6-well culture dishes

494 and treated with conditioned medium for 24h. RNA isolation was performed using Trizol
 495 (Life Technologies) or the RNeasy Kit (Qiagen) for MTMs. RT- and qPCR were
 496 performed as described ⁴¹. All samples were normalized to GAPDH expression and 2⁻
 497 $\Delta\Delta C_t$ values were calculated as described ⁴⁶. Primers were purchased from IDT.
 498 Primer sequences were: Mouse- GAPDH forward primer 5'-
 499 AACTTTGGCATTGTGGAAGGGCTC-3'; GAPDH reverse primer 5'-
 500 TGGAAGAGTGGGAGTTGCTGTTGA-3. E-Cadherin forward primer 5'-
 501 CAAGGACAGCCTTCTTTTCG-3'; E-Cadherin reverse primer 5'-
 502 TGGACTTCAGCGTCACTTTG-3'. Wnt-1 forward primer 5'-
 503 CAGTGGAAGGTGCAGTTGCAG-3'; Wnt-1 reverse primer 5'-
 504 CAGTGGAAGGTGCAGTTGCAGC-3'. CSF1 forward primer 5'-
 505 CAACAGCTTTGCTAAGTGCTCTA-3'; CSF1 reverse primer 5'-
 506 CACTGCTAGGGGTGGCTTTA-3'. CCL2 forward primer 5'-
 507 GGCTGGAGAGCTACAAGAGG-3'; CCL2 reverse primer 5'-
 508 GGTCAGCACAGACCTCTCTC-3'.

509 **Patient samples.** Paraffin embedded sections from patients with DCIS were obtained
 510 from the Cancer Biorepository at Icahn School of Medicine at Mount Sinai, New York,
 511 NY and from Thomas Karn, University of Frankfurt, Germany. Samples were fully de-
 512 identified and obtained with Institutional Review Board approval, which indicated that
 513 this work does not meet the definition of human subject research according to the 45
 514 CFR 46 and the Office of Human Subject Research.

Circulating Cancer Cells (CCCs) and Disseminated Cancer Cells (DCCs)

detection. For CCC analysis, blood was drawn by cardiac puncture following IACUC protocols. CCCs were purified using negative lineage cell-depletion kit (Miltenyi), fixed with 3% PFA for 20 min on ice and cytopsin preparations were carried out by centrifugation of blood cells at 500 rpm for 3 min using poly-L-lysine-coated slides (Sigma).

Statistical Analysis. Unless noted otherwise, all of the experiments presented in the manuscript were repeated at least 3 times with replicates of at least 3. Statistical Analysis was done using Graph Pad Prism Software. For all cell culture experiments (experiments with technical replicates), one-tailed *student t-tests* were performed. For mouse experiments, *Mann-Whitney* tests were used. Differences were considered significant if *P* values were ≤ 0.05 .

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Author Contributions.

N.L. designed and optimized experimental approach, performed *in vitro* and *in vivo* experiments, analyzed data and co-wrote the manuscript, A.M. and A.R. and the Human Immune Monitoring Core performed CyTOF experiments, E.F., M.S.S., E.T., and K.H. performed experiments, M.M. provided general guidance and oversight and co-wrote the manuscript, J.A.A.-G. designed and optimized experimental approach, provided general guidance and oversight, analyzed data and co-wrote the manuscript.

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Author information.

The authors declare no competing financial interests.

Figure Legends.

Figure 1: Macrophages enter the ductal epithelial layer in early breast cancer lesions. HE stainings of mammary gland sections show progression from healthy mammary ducts in FVB wild type glands (**A**) to early lesions classified as hyperplasia and mammary intra-epithelial neoplasia (**B**) to invasive tumors (**C**) in the MMTV-HER2 mouse model. Mammary glands from FVB wild type (**D**) of pre-malignant MMTV-HER2 mice at age 14 (**E**) and 22 (**F**) weeks were stained against F4/80 (macrophages) and CK8/18 (epithelial cells) and against F4/80 and SMA (**E-G**). Bars 10 μ m. The mean plus SEM of the percentage of ducts containing IEM is shown; each dot represents one animals (**H**).

Figure 2: Intra-epithelial macrophages induce an EMT-like response in early cancer cells. 20wk old MMTV-HER2 mouse mammary glands were stained against E-Cadherin and F4/80. E-Cadherin localization was analyzed dependent on whether macrophages did not make direct contact to the duct (no M. or distant M.; **A**) or whether ducts contain intra-epithelial macrophages (IEM; **B**). The percentage of individual epithelial cells that showed disrupted E-Cadherin (arrow in B) was quantified (**C**). Plot shows means plus SEM; each dot represents one animal. E-Cadherin mRNA expression in whole mammary glands of FVB wild type (WT) or 20wk old MMTV-HER2 mice was determined by qPCR. Plot (**D**) shows means plus SEM; each dot represents one animal. 24wk old MMTV-HER2 mammary glands were stained against β -Catenin and Iba1, a macrophage marker (**E,F**). β -Catenin⁺ early cancer cells (blue arrows in F)

were more frequent in ducts containing intra-epithelial macrophages (black arrows in F) (G). Each dot represents one animal, plots show mean plus SEM. The mammary epithelial cell line Comma-1D was grown as a monolayer and Raw264.7 macrophages stably transfected with mCherry were added. After 12h, co-cultures were stained against E-Cadherin (H) or β -Catenin (I) and the signal intensity of β -Catenin signals inside individual nuclei was quantified (J). Boxplot shows range of nuclear β -catenin signal intensity in individual cells; independent experiments N=3. (K,L) Conditioned medium was harvested from primary cultures of wild type (WT) or pre-malignant MMTV-HER2 mammospheres and added to Raw264.7 macrophages or mammary tissue macrophages (MTMs) isolated from pre-malignant MMTV-HER2 mammary glands. Wnt-1 expression was determined by qPCR, plots show means plus SEM of three technical replicates; individual experiments N=3 for Raw264.7 and N=2 for MTMs. Comma-1D cells were grown as monolayers (M) and Raw-264.7-mCherry macrophages were added (N) and additionally treated with DKK1 (O). Co-cultures were harvested after 12h and stained against E-Cadherin. E-Cadherin signal intensity in whole section was quantified. Plot (P) shows mean plus SEM; each dot represents one microscopic field; independent experiments N=2.

Figure 3: Macrophage depletion during pre-malignant stages prevents early cancer cell dissemination. 20wk old pre-malignant MMTV-HER2 mice were treated with the anti CSF1R ASF98 antibody and animals were harvested after two weeks with no signs of invasive carcinoma (A). Analysis of HE staining of mammary gland sections confirmed the absence of invasive lesions (B). E-Cadherin expression in whole

mammary glands was determined by RealTime PCR of whole mammary gland lysates (D; mean plus SEM; each dot represents one individual mammary glands; N=2 experiments combined) and by immunofluorescent staining against E-Cadherin in mammary gland sections (E,F; bars 10 μ m). E-Cadherin signal intensity was measured in regions of cell junctions (G). Box plots depict values of individual regions for 3 animals each; N=2. Early circulating cancer cells (eCCCs) were quantified by harvesting peripheral blood and determining the amount of HER2 and CK8/18 positive eCCs per mL blood which then were normalized to the mean of controls. Plot (H) depicts normalized means plus SEM; each dot represents one animal; combined independent experiments N=2. Disseminated eCCs were quantified by staining lung sections against HER2 (I,J; bars 25 μ M) and quantifying the average of HER2+ cells per 100 randomly chosen microscopic fields (K). Plot (K) shows means + SEM where each dot represents one animal of 2 individual experiments combined.

Figure 4: Early disseminated cancer cells can contribute to metastasis formation.

Macrophages were depleted from pre-malignant MMTV-HER2 mice by ASF98 treatment starting at week 18. Treatment was stopped when mice developed palpable tumors (1-3mm average) (A). Mice were left until tumors reached 1cm in diameter and then sacrificed. Time from beginning of treatment at age wk18 until development of palpable tumors (B) and from formation of palpable tumors until tumors were overt (C) is depicted as mean plus SEM where each dot represents one animal of two independent experiments combined. Macrophages in sections of overt tumors at the end of the experiment identified by staining against F4/80 (D,E) and quantified as

numbers of macrophages relative to tumor area (**F**). Vascularization of overt tumors was analyzed by staining against endomucin, an endothelial cell marker (**G,H**) and quantification of vascularized area (**I**). Plots show mean plus SEM where each dot represents one section of at least 3 animals combined. Solitary DCCs in lung sections and metastases defined as cell clusters bigger than 3 cells were quantified in lung sections stained against HER2 (**K**). For solitary cell analysis, the average of DCCs or metastases per 100 fields was counted; each dot represents one lung section (**J**). For metastasis analysis, the total number of metastases per lung sections was quantified and plotted (**L**). Number of mice N=6 (control) and N=4 (α CSF1R) animals combined; independent experiments N=2.

Figure 5: Phenotypic profiling of macrophages in early mammary cancer lesions.

Whole mammary glands from FVB wild type mice or 14 and 22 week old pre-malignant MMTV-HER2 mice were analyzed by mass cytometry. viSNE plots were generated from myelo-monocytic cells (gating strategy **Fig.5 supplement 1B**) (**A**). Results from one representative animal is shown; number of animals per group N=5; individual experiments N=2. The three subpopulations were identified as Ly6C⁺ monocytes and CD206^{hi} and CD206^{lo} macrophages based on their expression levels of Ly6C (**B**) and CD206 (**C**). These three populations were then analyzed for their frequency amongst all myelomonocytic cells (**D-F**) Dot plots show mean plus SEM of 5 animals per group, each dot represents one animal. Heat plots for 3 individual animals per group with expression levels of selected markers Ly6C (**B**), CD206 (**C**), Tie2 (**I**) and IdU incorporation as a proliferation marker (**J**) were generated for CD206^{lo} and ^{hi}

macrophages as identified in the viSNE plots. **G, H:** viSNE plot and quantification of myelomonocytic population in overt MMTV-HER2 tumors. Mammary glands from FVB wild type mice (**K**), MMTV-HER2 mice at 24wks (**L**) and overt MMTV-HER2 tumors (**M**) were stained against CD206 and F4/80 and CD206 signal intensity in F4/80+ macrophages was quantified. Plot (N) depicts mean plus EM, each dot represents one macrophages; 3 animals combined. Bars 10 μ m.

Figure 6: HER2 upregulation leads to activation of NFkB and CCL2 overexpression. Phosphorylation of the p65 subunit of NFkB was analyzed in Western Blots of whole cell lysates of mammospheres from 20wk old pre-malignant MMTV-HER2 mice (**A**). One representative plot of three independent experiments is shown. (**B**) CCL2 expression in mammospheres derived from FVB wild type and pre-malignant MMTV-HER2 mammary glands was measured by real time PCR analysis. Plot shows means plus SEM where each dot represents one technical replicate; number of individual experiments N=3. (**C**) Mammary glands from FVB wild type, 14wk old and 22wk old MMTV-HER2 mice were stained against CCL2, HER2, and CCR2. CCL2 immunofluorescent signal intensity around the ductal epithelium was quantified (**D**). Plot shows mean plus SEM, each dot represents one duct. Bars 25 μ m. CCL2 was stained in acini cultures derived from pre-malignant MMTV-HER2 mammary glands that were grown for 5d and then treated with DMSO (vehicle control; **E**), 1 μ M Lapatinib (**F**) or 1 μ M IKK inhibitor compound A (**G**) for 24h. Acini derived from 20 week old pre-malignant MMTV-HER2 mammary glands were grown for 5d, then treated with DMSO (vehicle control), Lapatinib (1 μ M), IKK inhibitor compound A (1 μ M), or CCR2 inhibitor RS504393

814 (1 μ M) and macrophages isolated from 20wk old pre-malignant MMTV-HER2 mammary
815 glands were added. After 24h, acini were stained against F4/80 and the percentage of
816 acini associated with mammary gland macrophages in 3D co-cultures (exemplary image
817 in **H**) compared to those not associated with macrophages (**I**) was determined (bar
818 25 μ m). Plot (**J**) shows means plus SEM; each dot represents one technical replicate;
819 independent experiments N=2. 20wk old pre-malignant MMTV-HER2 mice were treated
820 with a CCR2 inhibitor RS504393 (2mg/kg i.p. daily) for 2 weeks. Additionally, mice were
821 treated locally by injecting 2mg/kg CCR2 inhibitor into the fat pad of one gland and
822 vehicle control into the contra-lateral gland. Injections were performed daily for 5 days
823 (**N**). Pre-malignant mammary glands were stained against E-Cadherin and F4/80 (**K,L**,
824 bars 25 μ m) and intra-epithelial macrophage (IEM) containing ducts were quantified
825 (**M,O**). For local treatment, values were normalized to the IEM content of each contra-
826 lateral control treated gland. Plot shows means plus SEM; each dot represents one
827 animal.

828 **Figure 7: Intra-epithelial macrophage numbers in human DCIS lesions negatively**
829 **correlate with E-Cadherin levels.** Human adjacent healthy (**A**) and DCIS tissue (**B**)
830 was stained against CD68 (macrophages) and smooth muscle actin (SMA). Bar: 75 μ m.
831 Plot shows mean plus SEM of the percentage of ducts intra-epithelial macrophages
832 (IEM) from 7 healthy and 10 DCIS patients; each dot represents one patient (**C**).
833 Sections from human DCIS tissue were stained against CD68 (macrophages) and E-
834 Cadherin (**D,E**). E-Cadherin pixel intensity was quantified in regions of individual cell
835 junctions and medians for individual patients were quantified. Plot (**F**) depicts mean E-
836 Cadherin intensity throughout DCIS lesions of individual patients with low or high intra-

epithelial macrophage (IEM) numbers (total patient number N=12). All bars 25µm.
Scheme of macrophage (G) assisted early dissemination from pre-invasive lesions
where mammary tissue macrophages (MTM) are attracted into pre-invasive ducts by
early cancer cells (CC) via HER2 and NFκB mediated upregulation of CCL2. Intra-
epithelial macrophages secrete Wnt proteins and thereby induce an EMT that drives
early dissemination. Early disseminated cancer cells then contribute to metastasis
formation, either as a slow cycling seeds of metastasis (scenario 1) or by interacting
with the microenvironment to make it more permissive for the growth of more adapted
late cancer cells (scenario 2).

Supplementary Figure Legends.

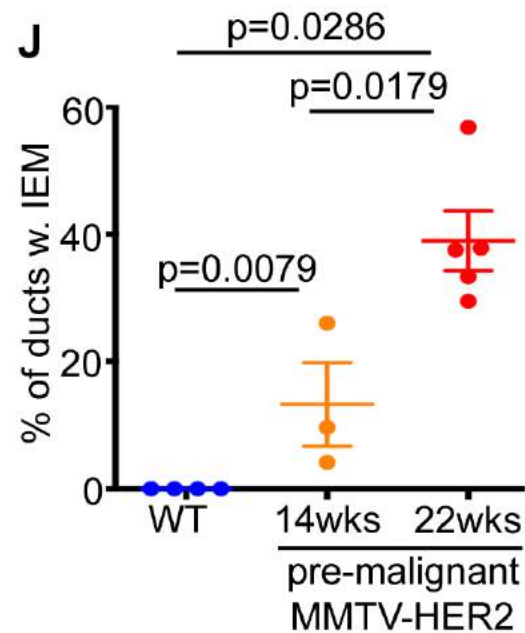
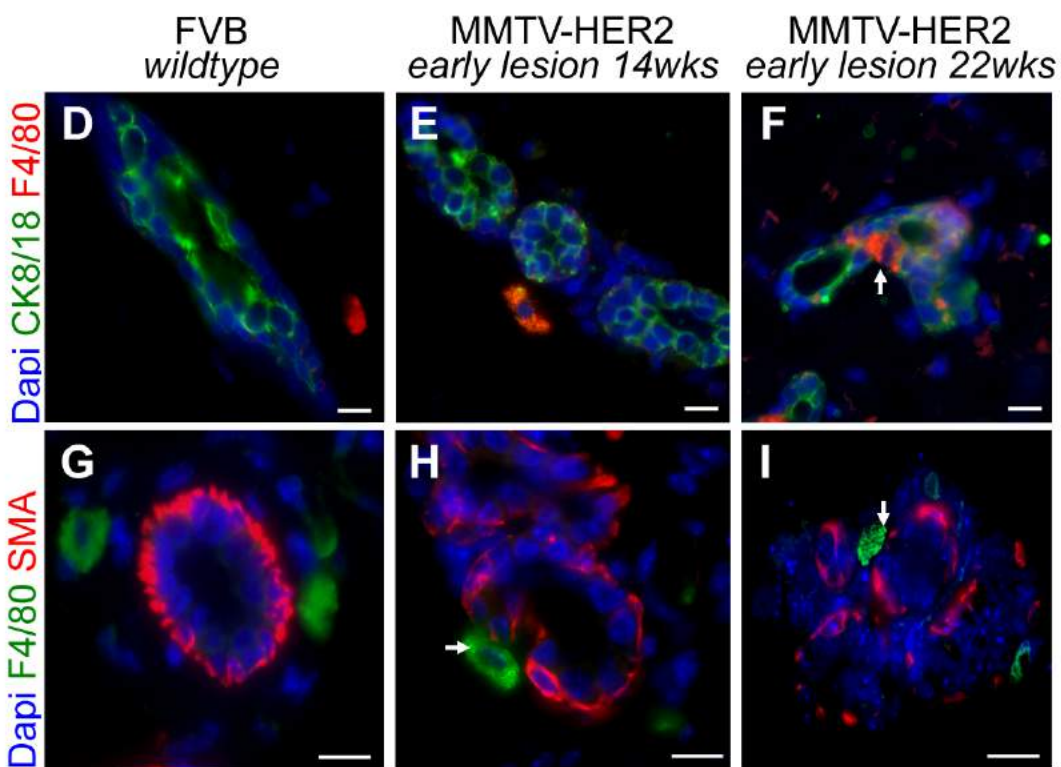
Supplementary Fig.1. Depletion of mammary gland macrophages (F4/80+/CD11b+) after two weeks of treatment with a CSF1R antibody compared to vehicle control (**A**). Percentage of ducts with intra-epithelial macrophages (IEM; **B**) and hyperplastic ducts (**C**) was quantified in mammary gland sections after two weeks of CSF1R antibody or vehicle treatment. Whole mount staining of mammary glands after two weeks of CSF1R antibody or vehicle control treatment (**D,E**).

Supplementary Fig.2. Flow cytometry lung gating strategy were CD45+ viable cells were identified as F4/80+/Gr1+/CD11b+ and F4/80-/Gr1+/CD11b+ monocytes and F4/80+/Gr1-/CD11c^{hi} alveolar macrophages (**A,B**). Quantification of alveolar macrophage (**C**) and monocyte populations (**D,E**) in lungs of MMTV-HER2 mice that had been treated with a CSF1R antibody or vehicle control during pre-malignant stages and were then allowed to progress to over tumor stages.

Supplementary Fig.3. A: List of markers analyzed by mass cytometry (CyTOF). **B:** Gating scheme to create viSNE plots of macrophages and monocytes. DNA staining and Cisplatin was used to identify viable cells. **C:** Heatplot of CD11b in the three myelo-monocytic populations identified by visne plot (Fig.2A). N=3 animals per group; independent experiments: N=2. **D:** Heatplot of VCAM1 in the three myelo-monocytic populations identified by viSNE plot. N=2 animals per group; independent experiments N=2.

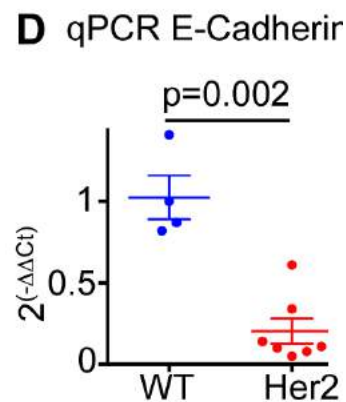
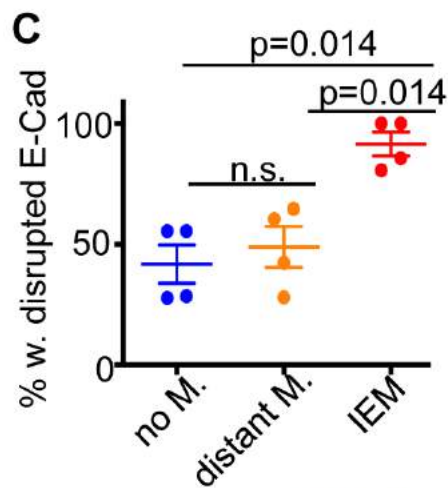
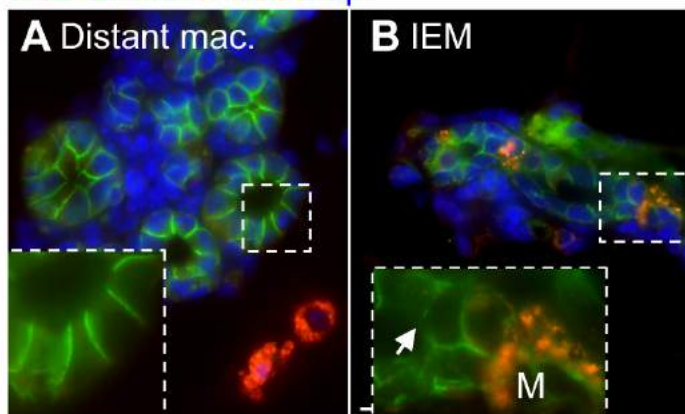
Supplementary Fig.4. A: Overview of qRT-PCR expression and immunofluorescent analysis results of selected cytokines in mammospheres derived from 20wk old MMTV-Her2 mammary glands that were not detectable (n.d.), did not change (n.c.) or did not change significantly (n.s.) or were increased in relation to expression levels in FVB wild type mammary glands. **B:** qRT-PCR expression analysis of CSF2 expression in mammospheres derived from either FVB wild type or 20wk old MMTV-Her2 pre-malignant mice. Three independent experiments with triplicates were performed. **C-E:** Immunofluorescent staining of FVB wild type (C) and 22wk old MMTV-Her2 mammary glands (D) against CSF2 or with IgG control (E). Bars 25µM

Supplementary Fig.5. Human DCIS sections were stained against CD68 and CD45 (**A**) and CD68 and cytokeratin 8/18 (**B**). **B,C:** Two individual ducts in one DCIS lesion of the same patient with either high E-Cadherin and low intra-epithelial macrophage (IEM) levels (**C**) or low E-Cadherin levels and high IEM levels (**D**) within the same patient. **E:** Quantification of E-Cadherin signal intensity in the region of cell junctions within ducts of the same patients with either high or low numbers of IEMs.

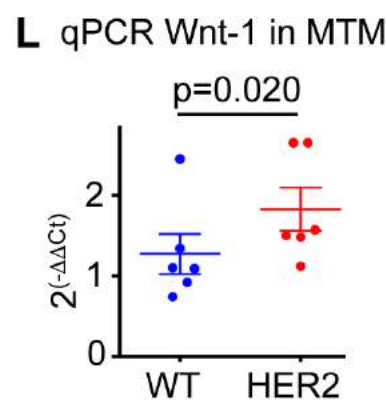
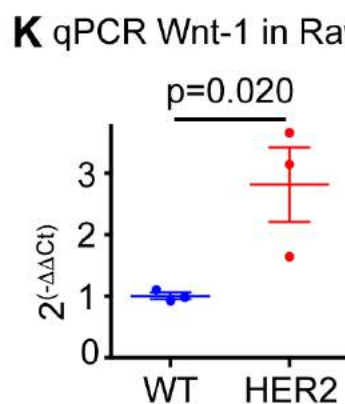
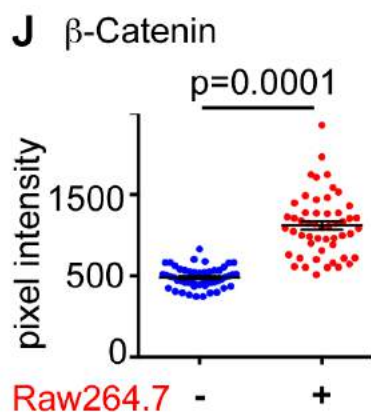
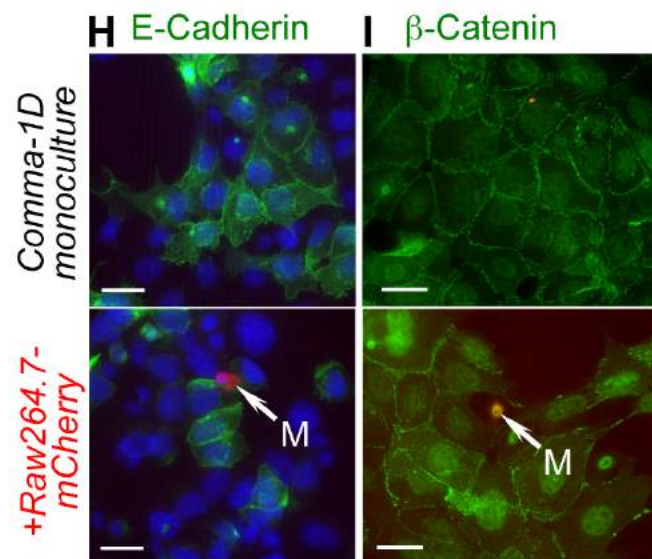
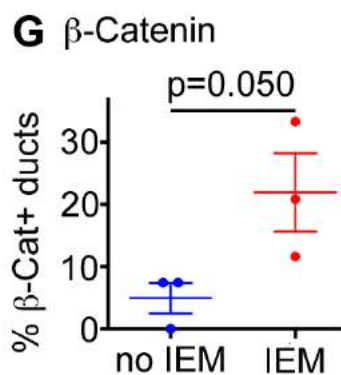
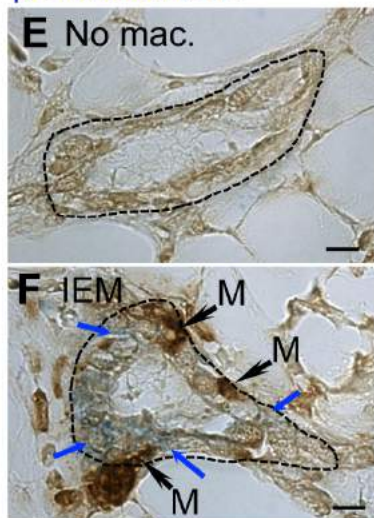


Linde et al. 2016 Figure 1

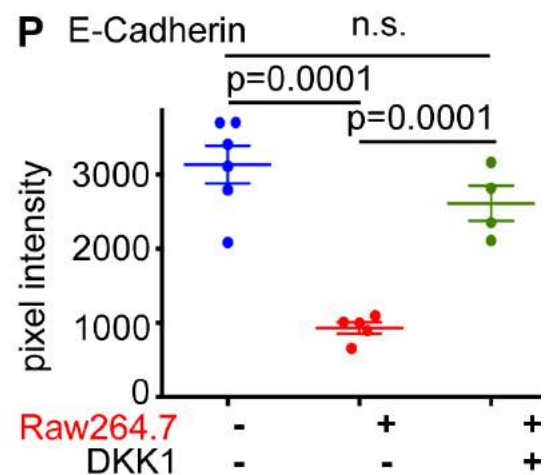
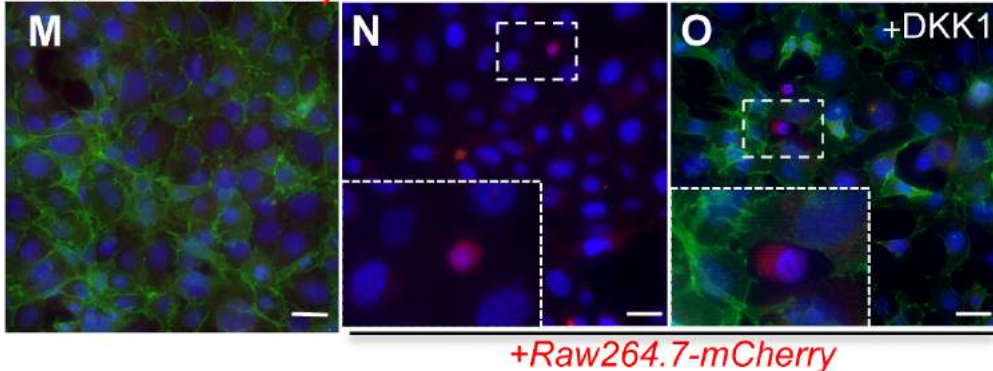
E-Cadherin F4/80 Dapi

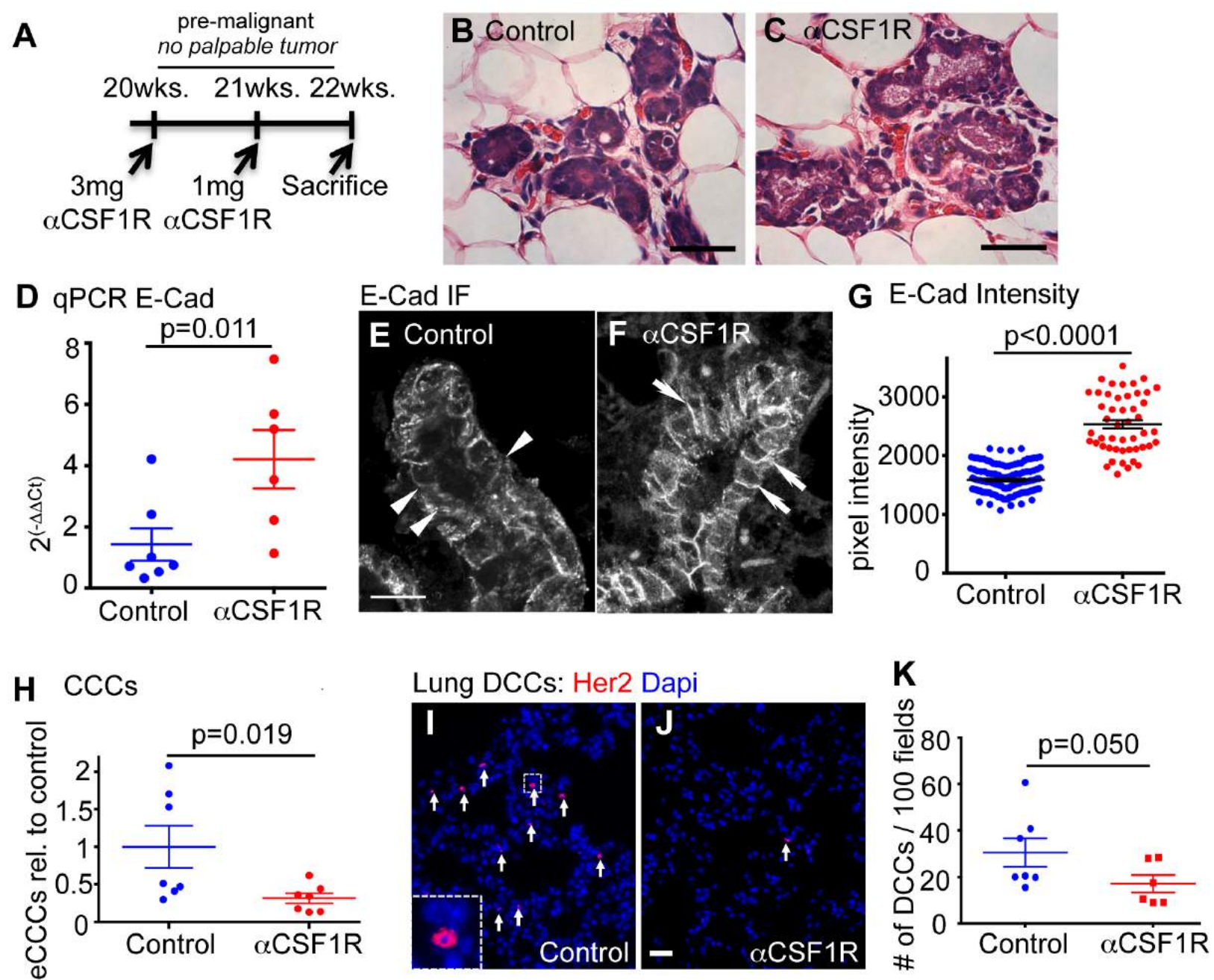


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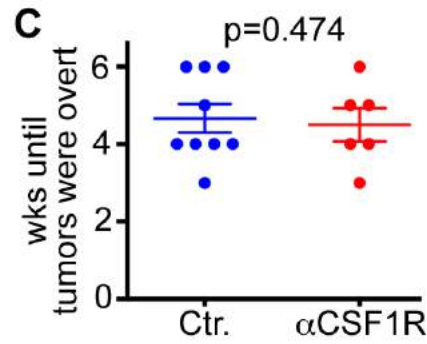
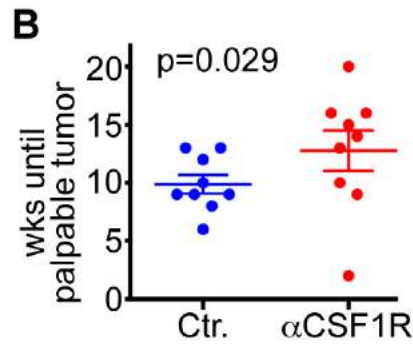
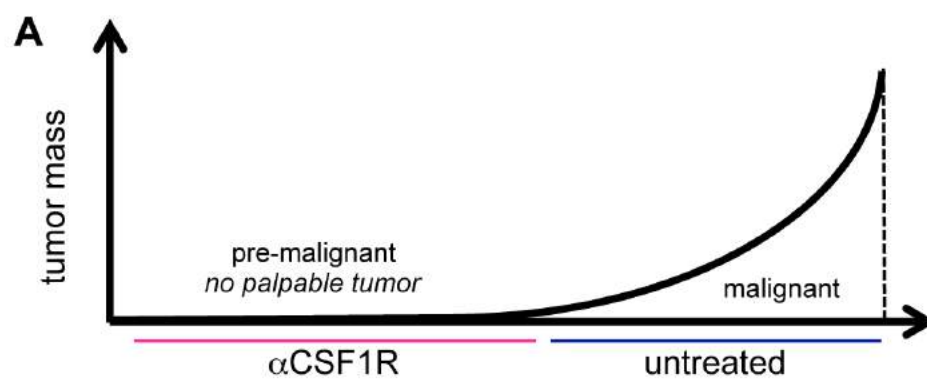


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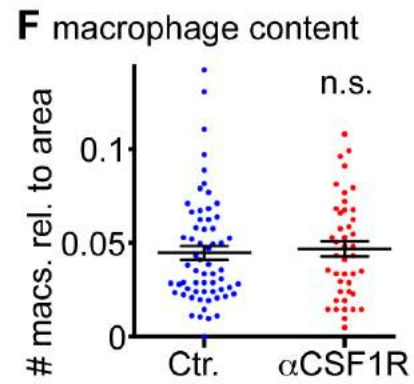
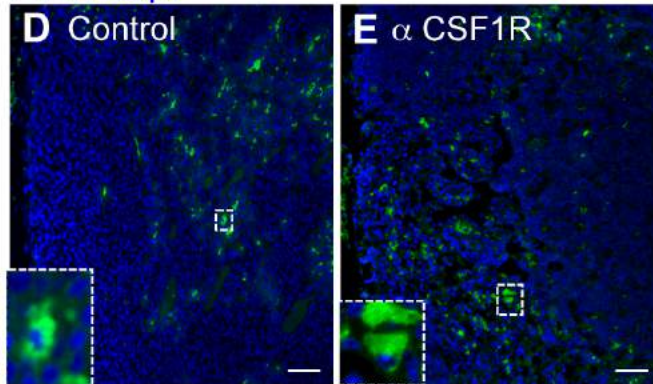




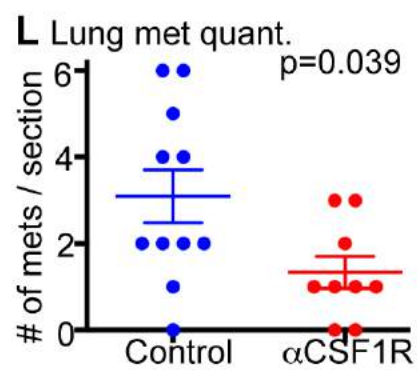
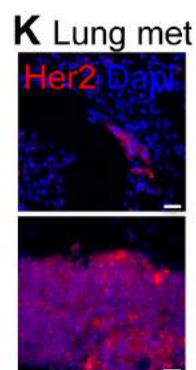
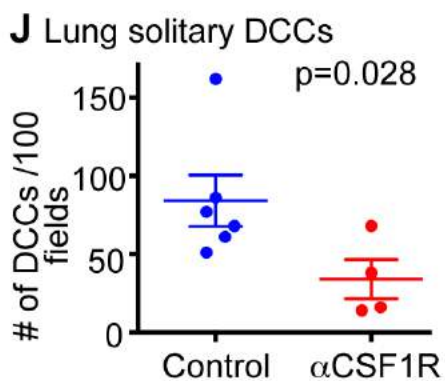
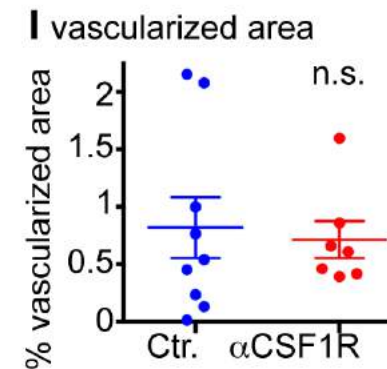
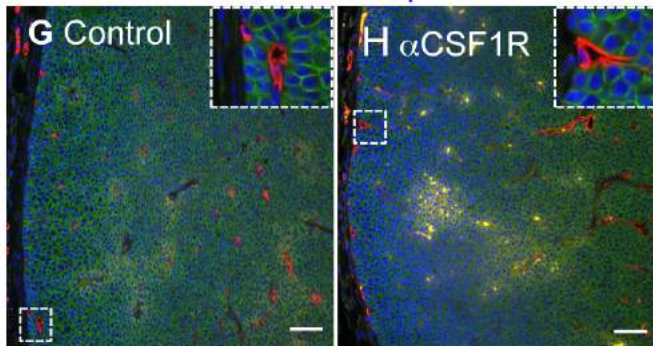
Linde et al. 2016 Figure 3

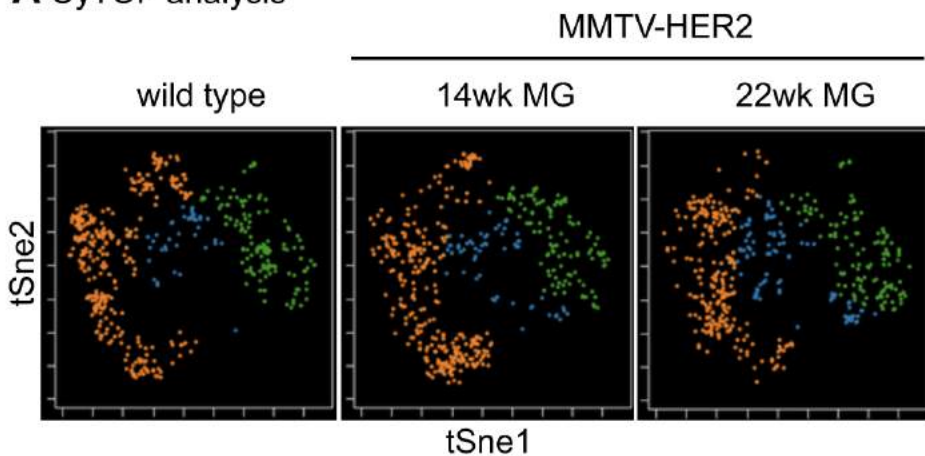
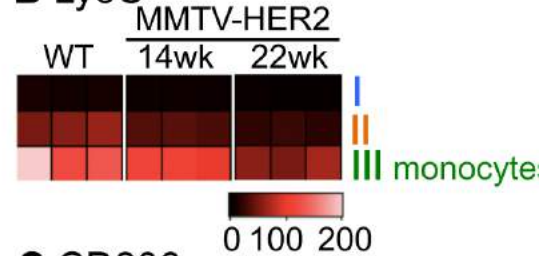
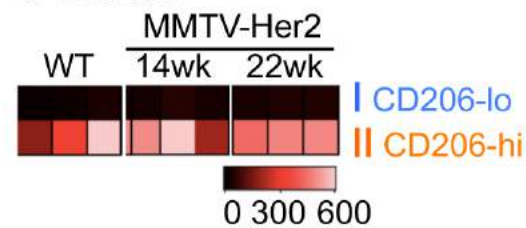
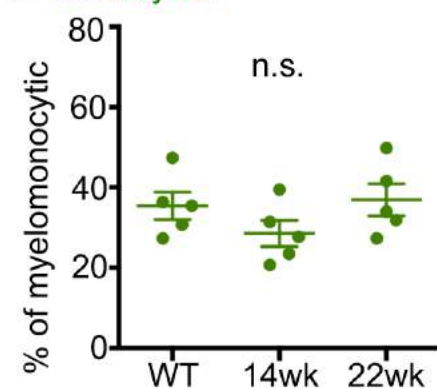
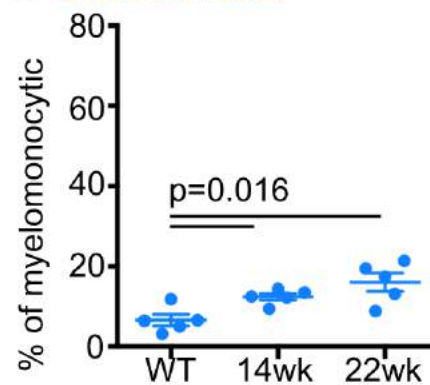
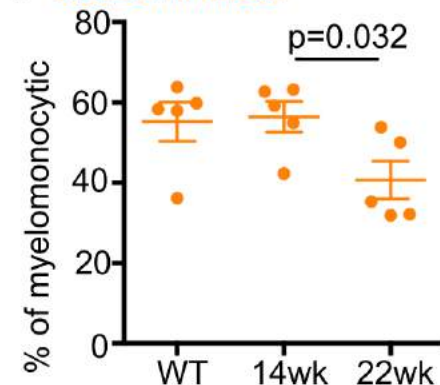
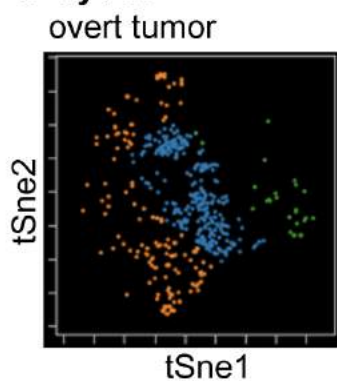
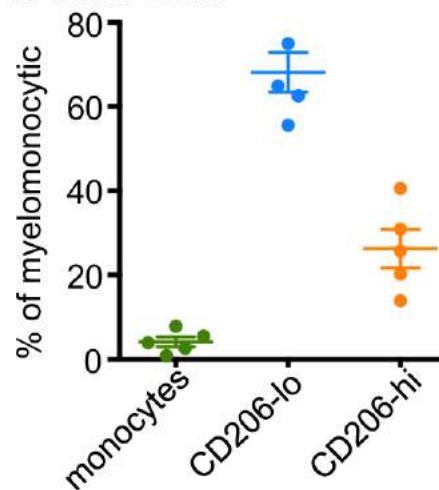
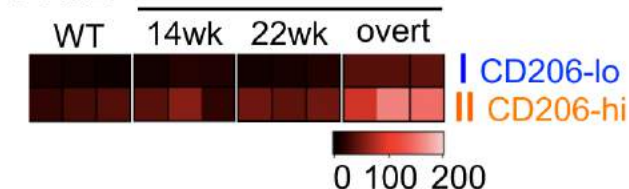
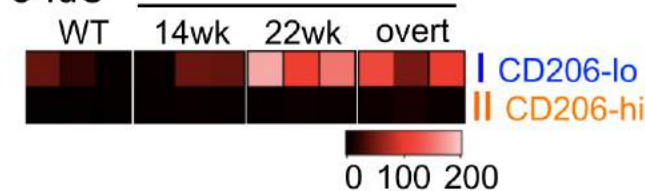
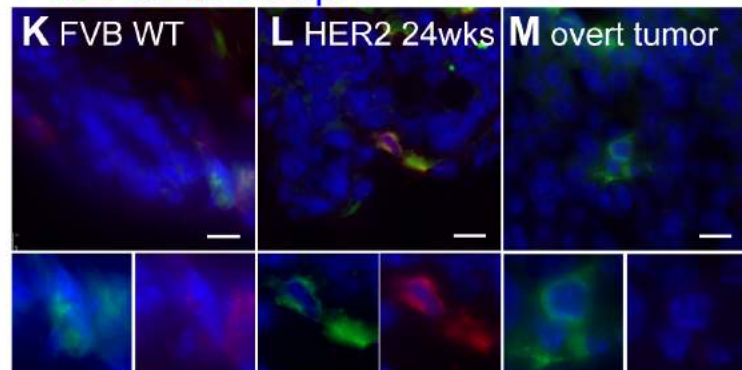
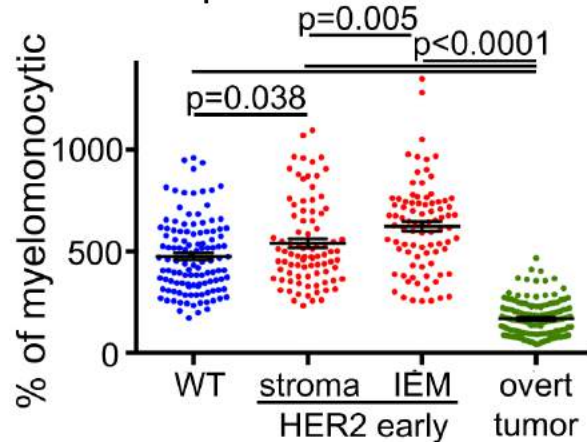


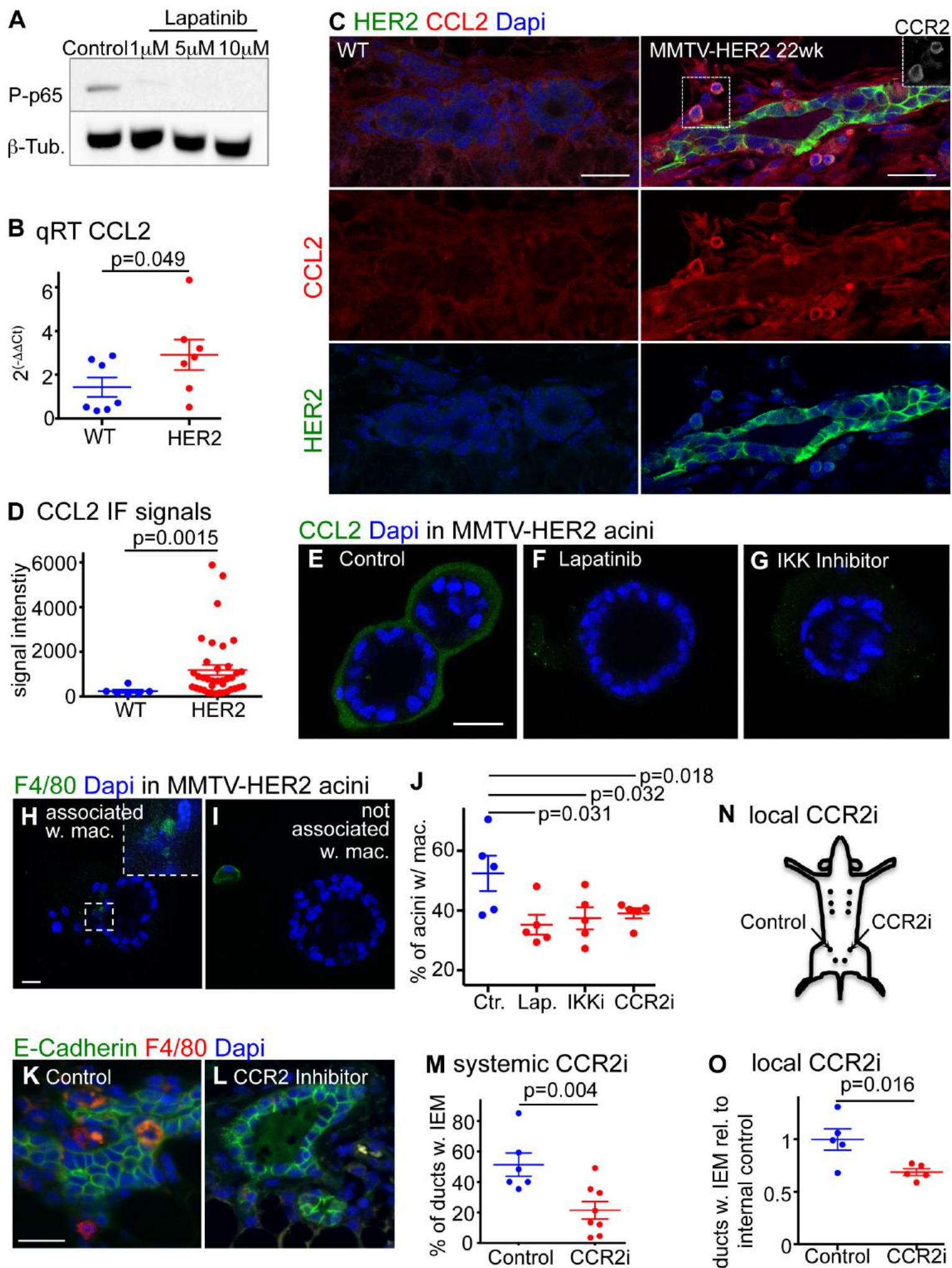
F4/80 Dapi

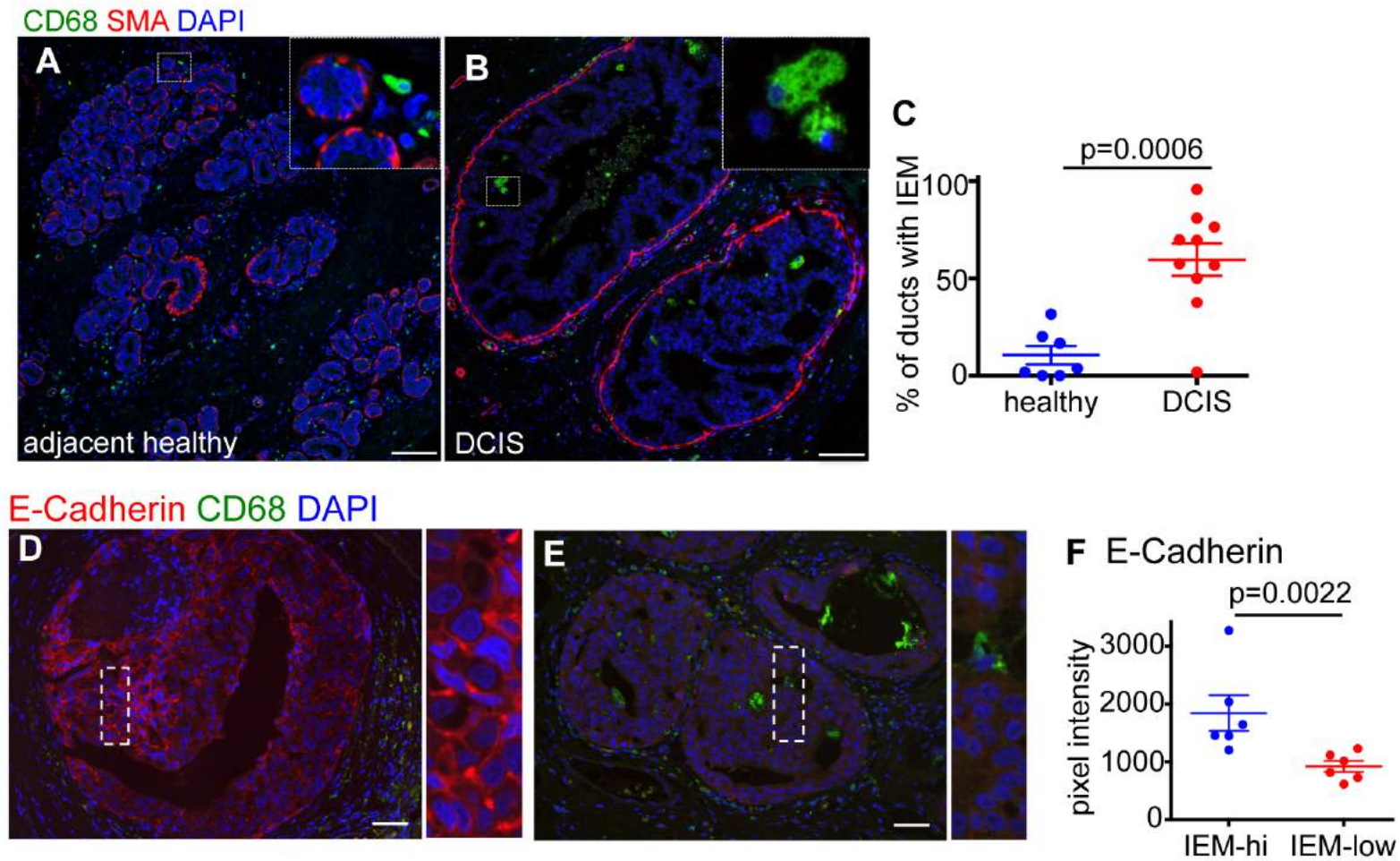


E-Cadherin Endomucin Dapi

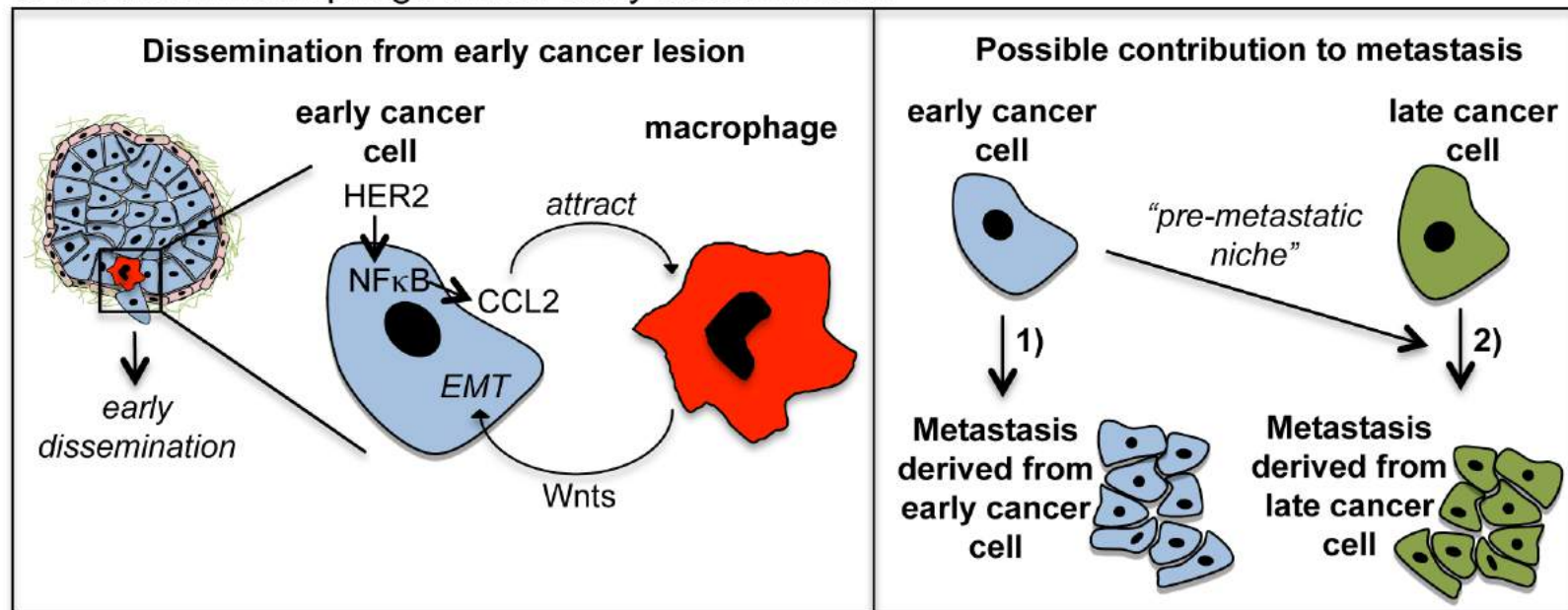


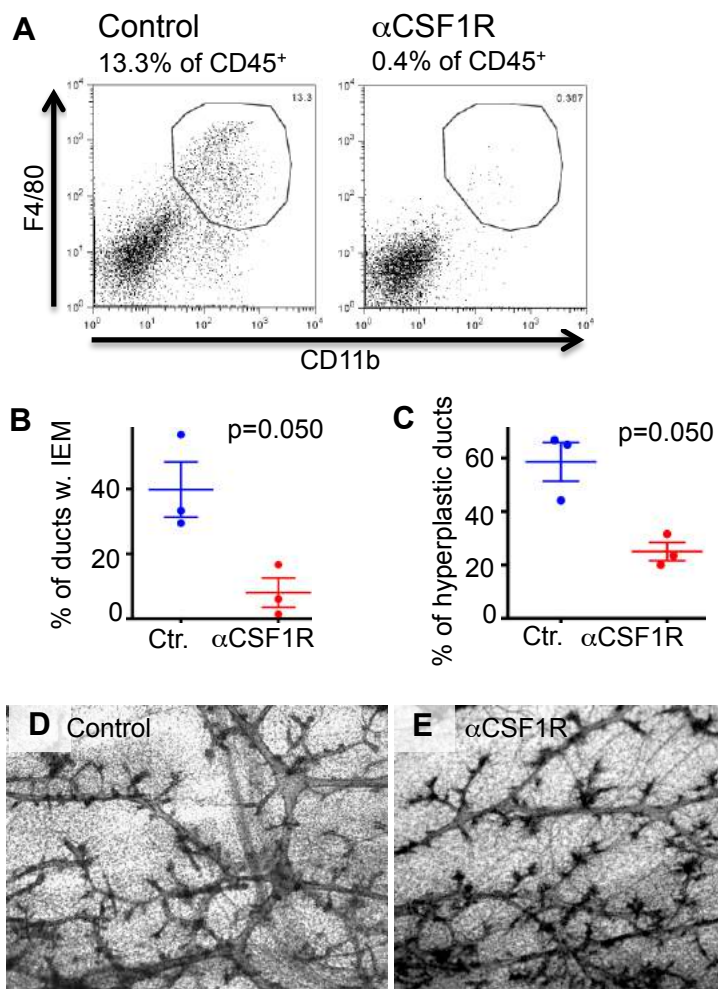
A CyTOF analysis**B** Ly6C**C** CD206**D** Monocytes**E** CD206-lo Macs.**F** CD206-hi Macs.**G** CyTOF**H** overt tumor**I** Tie2**J** IdU**K** F4/80 CD206 Dapi**N** CD206 quantification



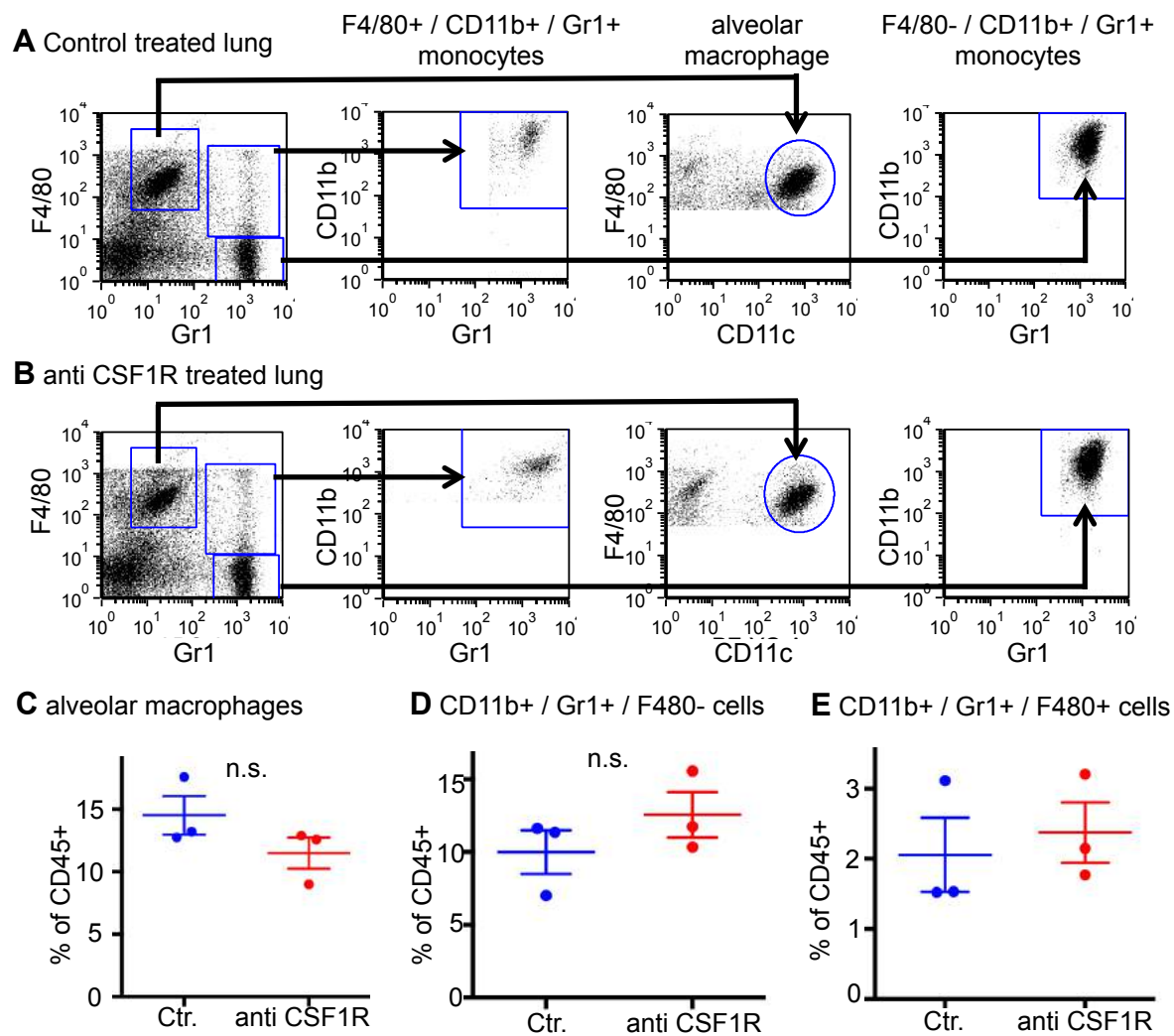


G Model of macrophage driven early dissemination

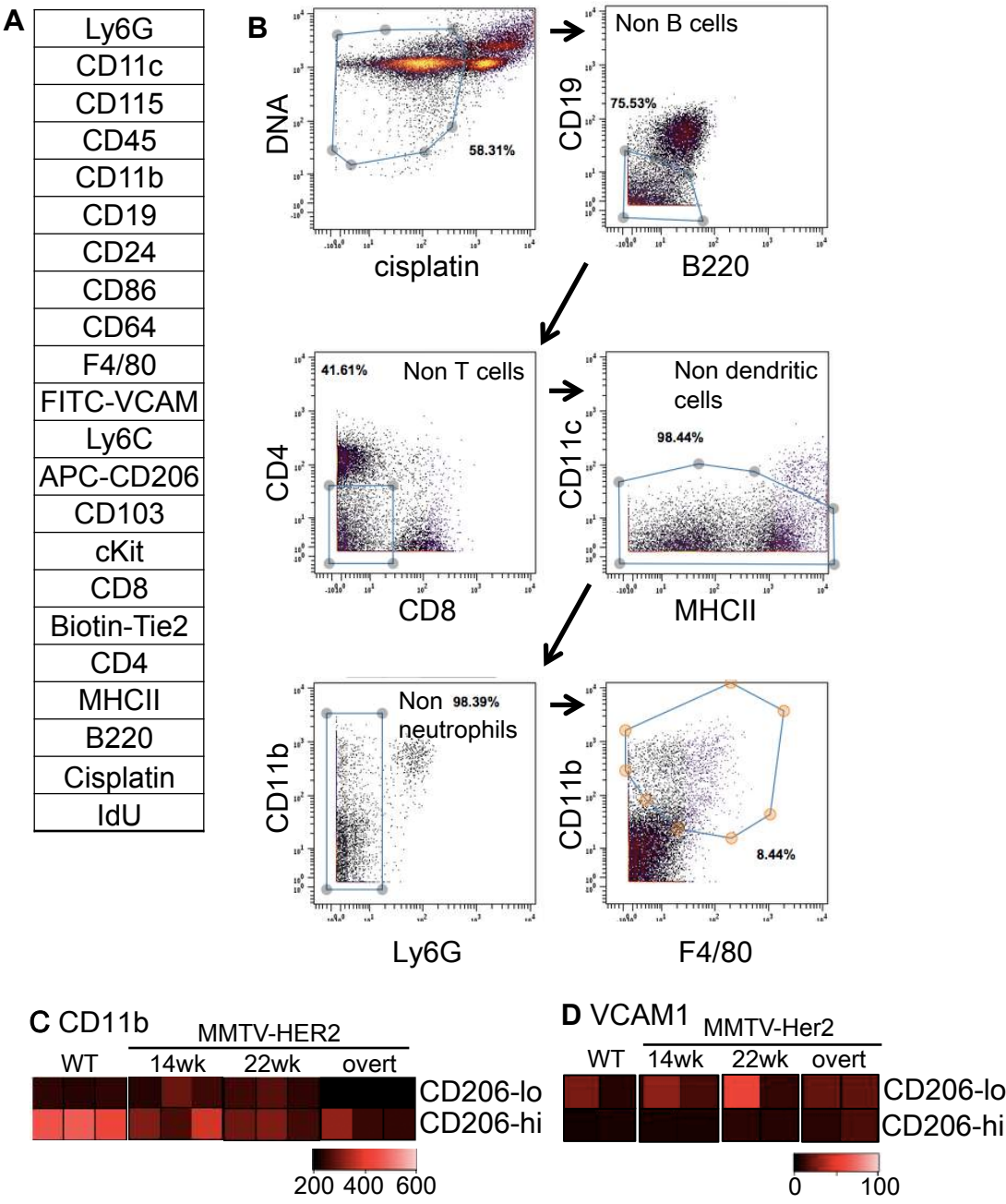




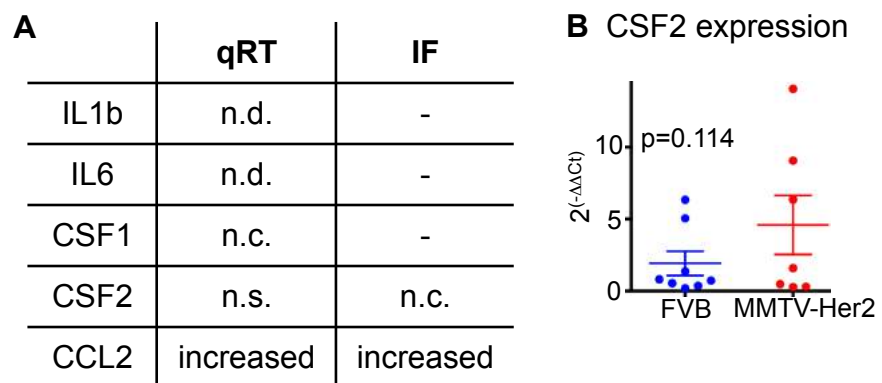
Linde et al. 2016 Supplementary Fig.1



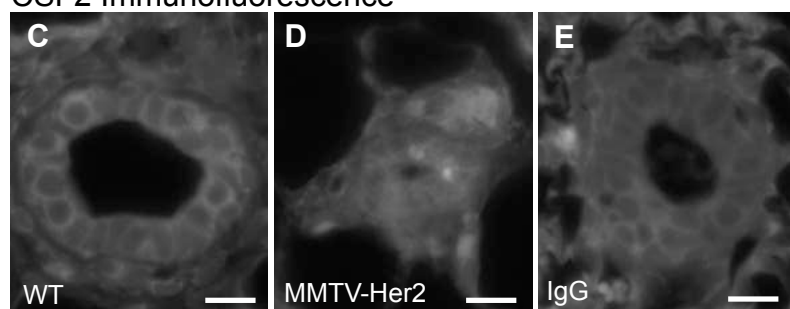
Linde et al. 2016 Supplementary Figure 2



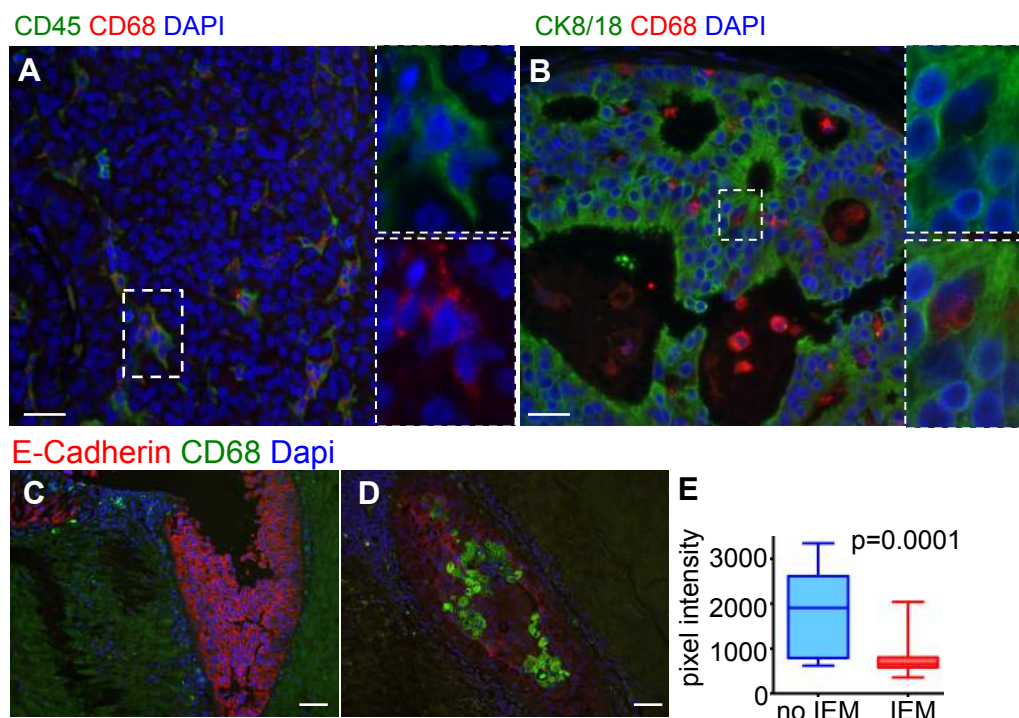
Linde et al. 2016 Supplementary Figure 3



CSF2 Immunofluorescence



Linde et al. 2016 Supplementary Figure 4



Linde et al. 2016 Supplementary Figure 5